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CHARACTERISTICS OF NITROGEN FIXATION IN MICROBIAL MATS FROM THE SOUTH TEXAS GULF COAST AND IN A CYANOBACTERIAL STRAIN ISOLATED FROM MATS

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CHARACTERISTICS OF NITROGEN FIXATION IN MICROBIAL MATS

FROM THE SOUTH TEXAS GULF COAST

AND IN A CYANOBACTERIAL STRAIN ISOLATED FROM MATS

Jingjie Yu, Ph.D.

The University of Texas at Austin, 2011

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Mature microbial mats from sandy intertidal beaches of the Texas Gulf coast demonstrated substantial levels of nitrogenase activity. Two species of non-heterocystous cyanobacteria, a *Hydrocoleum* and a *Microcoleus*, dominated the upper green layer of mature mats. Subsurface layers of cyanobacteria, but not mature mats, appeared during dry seasons. These "proto-mats" contained almost exclusively *Microcoleus* and demonstrated neither nitrogenase activity nor detectable nitrogenase reductase (Fe-protein). *Hydrocoleum*, identified from its morphology and 16s rDNA, was isolated and cultivated as unialgal cultures. Similar diel patterns of nitrogenase activity and Fe-protein expression were found in intact mature mats and in isolated *Hydrocoleum* cultures. Primers and a probe specific for the *Hydrocoleum nifH* gene, along with q-rtPCR measurements, demonstrated similar levels, but slightly different patterns, of expression in intact

mature mats and cultures of isolated *Hydrocoleum*. Increased levels of *nifH* transcripts and Fe-protein in *Hydrocoleum* cultures appeared before the end of the light period of a diel cycle, and the light period was required for nitrogenase activity in the subsequent dark period. Levels of *nifH* transcripts stayed very low and nitrogen fixation stopped when cultures were maintained under continuous darkness. The pattern of nitrogenase activity in *Hydrocoleum* cultures was not affected by elimination of ambient O_2, increasing or decreasing temperature in a range from 20 ˚C to 35 ˚C, or light intensity. However, the level of nitrogenase activity did vary with environmental conditions. Highest nitrogenase activities were observed when assays were conducted in an aerobic rather than an anaerobic environment, at 25 ˚C rather than a higher or lower temperature, and illuminated with bright (~ 900 $\mu E/m^2s^1$), rather than less intense light. Average levels of *nifH* transcripts were positively correlated with levels of nitrogenase activity.

Isolated cultures of *Hydrocoleum* formed mat-like structures in undisturbed flasks, suggesting that *Hydrocoleum* may be an early colonizer of intertidal sand for mat formation. However, observations of subsurface proto-mats indicate that *Microcoleus* is more likely to serve as the foundation for cyanobacterial mats, with *Hydrocoleum* later providing structural integrity and nitrogen availability. A process of successional development of microbial mats from the South Texas Gulf Coast is hypothesized.

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Abbreviations

BLAST	Basic Local Alignment Search Tool
Chl a	Chlorophyll a
CTAB	Cetyl trimethylammonium bromide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
EPS	Extra-cellular polymeric substances
NA	Nitrogenase activity
NCBI	National Center for Biotechnology Information
UTEX	The Culture Collection of Algae at the University of Texas at Austin

CHAPTER ONE: INTRODUCTION

A. Introduction to microbial mats

Microbial mats are small-scale ecosystems. They are vertically layered structures that include stratified groups of microorganisms with diverse functions within these layers. The earliest microbial mats date back at least 3.5 billion years. Fossils of these ancient structures, called “stromatolites”, are one of the strongest lines of evidence of early life on Earth. Extensive microbial mats occurred during the Proterozoic eon. They, along with phytoplankton, are believed to be responsible for most initial primary production in the world (Staley 2010). Photosynthetic microbial mats may also have contributed to the formation of the CO_2-limited and O_2 abundant atmosphere (des Marais 2003, Krumbein et al. 2003).

Microbial mats are still widespread around the world, existing in various kinds of environment conditions. They have been reported not only from quite normal conditions, such as environments around lakes and rivers, or in transitional environments (Stal 2001) such as intertidal flats (Kremer et al. 2008), but also from extreme conditions, such as thermal springs (Ward et al. 2006, Steunou et al. 2008), hyper-saline springs (des Marais 2003, Ley et al. 2006), Artic or Antarctic areas and deserts (Bottos et al. 2008, Jungblut and Neilan 2009, Zeglin et al. 2009). The complex community organization of mats makes them well suited for a diverse range of research. For example, studies of Archaea and

bacteria occurring in hot spring mats provide information pertaining to evolutionary process of microorganisms since the hyperthermal environment is the setting for the early Earth. Cyanobacteria from mats in polar region serve as models for research of stress response and adaption of microorganisms during cooling and freeze-events.

The diversity of organisms in microbial mats varies according to the location and type of mats. Common dominant groups in fully developed mats include cyanobacteria, colorless sulfur bacteria, purple sulfur bacteria, and sulfate-reducing bacteria (van Gemerden 1993, Gerdes 2010). Other kinds of microorganisms are also detected in various specific kinds of mats. Heterotrophic bacteria, such as α-, β-, and γ-proteobacteria are pervasive in nitrogen fixing mats (Zehr et al. 1995); chemolithotrophic gliding bacteria (such as *Thioploca*) form mats in oxygen-depleted mud (Oschmann 2000); Anaerobic methane oxidizing Archaea dominate microbial mats from the black sea (Michaelis et al. 2002, Reitner et al. 2005). Although microbial mats are mostly composed of prokaryotic organisms, they also occasionally contain eukaryotic organisms, such as diatoms, nematodes, or fungal mycelia (Verrecchia 2000, Sabbe et al. 2004, Feazel et al. 2008).

In the “joint ventures” theory of mat formation and expansion (van Gemerden 1993), microbial mats have the tendency spread both horizontally and upward whenever the moisture is available. Motile organisms can migrate via particulate matter surrounding them. Due to upward migration, older organic matter

becomes buried deeper and deeper, and finally becomes degraded. The colorful stratification, which is a striking feature of many mats, is considered to be a side-effect of upward-directed growth under varying conditions, biomass in various states of decay, and stratified, diverse metabolic activity. For example, the top layer of green surfaces of mats usually is composed of cyanobacteria, a colored intermediate layer (if available) is dominated by purple phototrophic bacteria (e.g. *Chromatium*, *Chloroflexus*), and lower layers, often black in color, results from the activity of sulfate-reducing bacteria (e.g. *Desulfonema*). Schulz (Schulz 1937) created a term “Farbstreifen-Sandwatt” (versicolored sandy tidal flats) to describe this colored stratification of mats.

Organisms in microbial mats are believed to distribute spatially according to oxygen and sulfide gradients as well as their metabolic potential. Aerobic phototrophic and heterotrophic bacteria occur in the surface layers of mats, away from anoxic and sulfidic environments; colorless sulfur bacteria are found at the base of the oxygen chemocline at the interface between oxygen-rich and sulfide-dominant layers; anoxygenic phototrophic bacteria occur below the oxygen chemocline if light of appropriate wavelengths can penetrate; sulfate-reducing bacteria and sometimes methanogens, may be found even lower where neither oxygen nor light are available (van Gemerden 1993, des Marais 2003).

B. Cyanobacteria in microbial mats

Cyanobacteria have been reported to be the dominant organisms in microbial mats in various environments (Severin and Stal 2010a). One probable reason is that many cyanobacteria have the ability to secrete a mucilaginous substance, forming a matrix that incorporates other organisms and materials into units that have structural integrity, stability and cohesiveness. The mucilage, described as extra-cellular polymeric substances (EPS), is composed of highly organized polymers like cellulose, and small molecules such as uronic acids and amino acids (Decho 2000). Because of their ability to secrete EPS, cyanobacteria are considered to be the founders that are responsible for early colonization leading to the establishment of microbial mats. In some well-studied stromatolite samples, cohesion provided by EPS contributed significantly to the formation of the organic framework (Verrecchia et al. 1995).

Cyanobacteria are oxygenic photosynthetic organisms, some of which are capable of nitrogen fixation. These two features allow cyanobacteria to play important, often dominant, roles in microbial mats. Cyanobacteria-based mats often thrive in extreme and rapidly fluctuating environments. These conditions are often accompanied by nutritional limitations that preclude most potential grazers. Cyanobacteria have low nutritional requirements because of their ability to capture CO_2 (and in some cases N_2) from the atmosphere. The ability to metabolize and assimilate these two compounds provides a cyanobacterium with

theoretically unlimited access to the two elements that are quantitatively most important for all living biomass.

Cyanobacterial photosynthesis supports the carbon needs not only of the cyanobacterium, but also is the driving force for carbon cycling in the entire microbial mat. Some water-soluble organic compounds (e.g. glycogen, acetate, lactate and ethanol) are released from cyanobacteria into the matrix of the mat, where they become available to heterotrophic bacteria (Anderson et al. 1987, Jørgensen et al. 1992, Stal 1995, Nold and Ward 1996, Stal and Moezelaar 1997). Other organic substances are secreted as EPS. Conditions such as high salinity, desiccation and limiting nutrition stimulate the synthesis of EPS (Myklestad et al. 1989, Staats et al. 2000), which then become an alternative source of carbon (Decho 1990, Flemming and Wingender 2001) for heterotrophic bacteria in the community.

Cyanobacterial nitrogen fixation can also be advantageous to the cyanobacterium and to an entire mat that includes the cyanobacterium, especially in nitrogen-depleted environments. Details of nitrogen fixation will be described in sections D and E.

Cyanobacteria are the dominant organisms of most microbial mats in the world, in part due to their ability to capture atmospheric carbon (and perhaps nitrogen), and their structural characteristics, but also due to their ability to adapt to extreme and rapidly fluctuating environmental conditions. The following examples illustrate their tolerance for diverse conditions. (a). *Microcoleus*

chthonoplastes, a filamentous non-heterocystous cyanobacterium, can live in a wide range of salinities, from fresh water to 100 ‰ (Karsten 1996, Nubel et al. 2000). It has been reported to be the predominant organism in a variety of different hypersaline microbial mats (Prufert-Bebout and Garcia-Pichel 1994). *M. chthonoplastes* also has the ability to perform sulfide-insensitive oxygenic photosynthesis at the same time as sulfide-dependent anoxygenic photosynthesis (Cohen et al. 1986, Cohen 1989). It is able to move, to do fermentation and elemental sulfur reduction (Whale and Walsby 1984, Garcia-Pichel and Prufert-Bebout 1996, Moezelaar et al. 1996). It produces a mycosporine-like amino acid that protects itself from UV-B damage (Karsten 2002). This combination of characteristics allows *Microcoleus* to survive and become abundant in environments that are highly fluctuating and toxic to many potential grazers. (b). Several species of *Synechococcus* are found in thermal to hyperthermal springs of Yellowstone National Park. They are believed to have all descended from a recent common ancestor. As a result of adaptive radiation, *Synochococcus* now dominates various kinds of microbial mats in the park, including those with optimal growth rates at diverse (from 42 °C to 74 °C) temperatures (Ward et al. 1998). (c). Common mat-dominant cyanobacteria such as *Oscillatoria* and *Phormidium* are capable of degrading some petroleum compounds, and directly contribute to the uptake and oxidation of n-alkanes. They are found in heavily polluted microbial mats (Abed et al. 2002), and are valuable for cleaning up oil-contaminated environments (Al-Hasan et al. 1998).

Filamentous non-heterocystous cyanobacteria that have been frequently detected in microbial mats include species in the Order Oscillatoriales, such as *Microcoleus*, *Oscillatoria*, *Lygnbya*, *Phormidium* and *Hydrocoleum* (Zehr et al. 1995, Paerl et al. 1996, Omoregie et al. 2004, Charpy 2007). Heterocystous genera (Order Nostocales) found in mats include *Nostoc* and *Nodularia* (Jungblut et al. 2005, Charpy 2007). Some unicellular cyanobacteria, such as *Gloeothece*, *Synechocystis* and *Synechococcus* (Steunou et al. 2008, Severin and Stal 2010b) also are often found in microbial mats.

C. A common cyanobacterium in tropic microbial mats – *Hydrocoleum*

Hydrocoleum is a filamentous non-heterocystous cyanobacterium belonging to the order of Oscillatoriales. It was first described by Gomont (Gomont 1892). Major morphologic characteristics of *Hydrocoleum* include: (a). Filaments usually joined in flattened mats with gelatinous and longitudinally stratified lamellate sheaths. (b). Trichomes cylindrical, unconstricted or slightly constricted at the cross walls, and usually motile within sheaths. (c). End cells of trichomes are sometimes with thickened outer cell wall or with narrow calyptra (Komárek and Hauer 2011). *Hydrocoleum* is considered one of the most common mat-forming cyanobacteria in tropical oceans. It is morphologically and phylogenetically similar to planktonic species of *Trichodesmium* (Abed et al. 2006). *Hydrocoleum* species from tropic microbial communities have been reported to possess the potential of nitrogen fixation (Abed et al. 2006). However, nitrogen fixation by

Hydrocoleum has not been convincingly demonstrated in isolated cultures or within microbial mats.

D. Nitrogen fixation in microbial mats

The element nitrogen (N) is essential to all organisms (Francis et al. 2007). As an essential component of proteins, nitrogen is fundamental to both structures and biochemical processes of life. The largest reservoir of N on Earth is N_2 gas (78% of the atmosphere). However, most organisms cannot directly use this triple-bonded form of N. N_2 molecules must be fixed (converted to non-volatile compounds) by diazotrophic microorganisms before they can be used by other organisms in the global N cycle.

The importance of biological nitrogen fixation to the global nitrogen budget, especially in marine environments, has been demonstrated in many studies (Gruber and Sarmiento 1997, Karl et al. 2002). Nitrogen fixed by free-living microorganisms or microbial communities in the subtropical Atlantic and Pacific oceans is believed to supply more than half of the total nitrogen demand of these areas (Karl et al. 1997, Carpenter et al. 1999, Dore et al. 2002) As one of the most widespread microbial communities on Earth, microbial mats have attracted much attention. Studies of nitrogen fixation in microbial mats have shown that the nitrogen fixed by microorganisms in mats not only supports primary production of these communities (Charpy et al. 2009), but also becomes a significant source of nutrition to other nearby ecosystems (Joye and Lee 2004).

Nitrogen fixation in microbial mats is facilitated by diazotrophic prokaryotes. Studies of the diversity of these organisms usually focus on identifying nitrogenase genes rather than characterizing isolated cultures, since less than 10% of the diazotrophs in microbial assemblages can be cultivated (Guerinot and Colwell 1985). Nitrogenase genes are well conserved although they are found in a large variety of phylogenetically diverse diazotrophic microorganisms, including free-living organisms and symbiotic species that are tightly associated with plants (Hennecke et al. 1985). A result of studies of many different N_2-fixing organisms in various habitats has been the development of an extensive database of nitrogenase genes. It is becoming one of the largest non-ribosomal gene datasets for uncultivated microorganisms (Zehr et al. 2003). Based on this database, the diversity of diazotrophs in various kinds of microbial mats has been examined. Besides cyanobacteria, which are often proposed to be the most important diazotrophic organisms in mats (Paerl et al. 1991, Bebout et al. 1993), many different aerobic and anaerobic heterotrophic bacteria (α-, β-, γ- and δ-proteobacteria) have the potential to fix nitrogen in microbial mats (Zehr et al. 1995, Zehr et al. 2003). RNA expression has been used recently to identify organisms that probably contribute to nitrogen fixation activity of mat communities, since the occurrence of nitrogenase genes does not demonstrate that they function (Zani et al. 2000, Steppe and Paerl 2005). However, these authors demonstrated that in most cases, N_2-fixation activity is correlated with the

occurrence of the corresponding genes, which means nitrogen fixation in microbial mats is the combination of activities from various groups of diazotrophs.

The diazotrophic community composition of a microbial mat changes according to environmental conditions, such as temperature, light availability, oxygen penetration, etc. As a result of this change, N_2-fixing activity of the mat exhibits seasonal dynamics (Zehr et al. 1995, Paerl et al. 1996, Severin and Stal 2010c). It is thought that different groups of diazotrophic organisms have different strategies for fixing nitrogen. Conditions that favor one diazotrophic group may not be optimal for another group (Severin and Stal 2010a). In studies of an intertidal mat, winter environment favored chemotrophic N_2-fixing organisms rather than cyanobacteria, so the nitrogen fixation pattern of the mat peaked during the night in summer but shifted to the middle of a day in winter (Paerl et al. 1996). *Lyngbya* was the dominant cyanobacterium in the mat from spring to fall, but it and its nitrogenase gene were undetected in winter (Zehr et al. 1995).

Even though the N_2-fixing activity of mats may be determined by various kinds of diazotrophic organisms, the pattern of activity and the major type of N-fixing cyanobacteria in microbial mats can sometimes be correlated. For example, mats dominated by heterocystous cyanobacteria had high N_2-fixing activity in daytime (Charpy 2007); mats with high percentage of non-heterocystous cyanobacteria showed nighttime N_2-fixing activity (Diaz et al. 1990, Charpy 2007). When both heterocystous and non-heterocystous cyanobacteria were abundant in mats, N_2-fixing activity was detected in both daytime and night time (Charpy-Roubaud et al.

2001, Charpy-Roubaud and Larkum 2005). These correlations are relatively general, but exceptions have been found. A mat that was dominated by non-heterocystous cyanobacteria showed higher daytime activity than nighttime activity (Bauer et al. 2008). This and other similar observations raise the prospect that cyanobacteria may not necessarily represent the major diazotrophs in microbial mats. Under some conditions, other diazotrophic organisms such as heterotrophic bacteria in mats may assume a dominant N_2-fixing role even though cyanobacteria remain morphologically dominant. If that hypothesis is true, then the N_2-fixing pattern of mats cannot be simply correlated with the type of cyanobacteria present. In order to determine which organisms are primarily responsible for the N_2-fixing activity in cyanobacteria-based mat, investigations that focus on individual species are required. Comparative studies of nitrogen fixation in mats and in isolated cultures of prospective nitrogen-fixing species obtained from those mats would be especially useful, but have not been reported.

E. Nitrogenase activity and regulation

Nitrogen fixation is catalyzed by nitrogenase, an enzyme complex consisting of two kinds of subunits: Mo-Fe protein (dinitrogenase) and Fe-protein (dinitrogenase reductase) (Orme-Johnson 1992). Mo-Fe protein is an $\alpha_2\beta_2$ tetramer (250 KD) and Fe-protein is a dimer (70 KD) of two identical subunits (Postgate 1982). The α and β subunits of the Mo-Fe protein, and the two subunits of Fe-protein, are encoded respectively by the *nifD*, *nifK* and *nifH* genes.

These three genes are on the same operon. Some organisms have “alternative” or “second alternative” nitrogenases where Mo is replaced by V (*vnfH*) or Fe (*anfH*) in the Fe-S center that bridges the subunits (Burgess and Lowe 1996, Eady 1996). Besides these structural genes, a number of other genes are required for regulation and assembly of nitrogenase, and the total length of DNA that is involved in the nitrogen fixation can reach 20 kb. Nitrogenase gene expression is highly regulated from the transcriptional level to the post-translational level (Chen et al. 1998) (Kim et al. 1999, Hoover 2000), but transcription of the *nifHDK* operon is still considered to be a meaningful indicator of nitrogen fixation, since it is not constitutively expressed and is modulated in response to factors that control N_2-fixation (Zehr et al. 2003). Within this operon *nifH* expression is measured most frequently as a monitor of probable nitrogenase activity.

Nitrogen fixation has a high demand for energy and reducing units, consuming 16 ATP and 8 electrons for each molecule of N_2 that is reduced to the level of NH_3. The nitrogenase enzyme complex is very sensitive to oxygen and its activity is irreversibly inhibited by oxygen *in vitro* (Berman-Frank et al. 2001). Many studies and interpretations of nitrogen fixation by diazotrophic organisms or microbial mats have focused on two critical factors: energy availability and oxygen concentration. Other factors such as the presence of combined nitrogen sources, the availability of phosphorus or metal ions, and temperature are also considered to be important (Paerl et al. 1987, Karl et al. 2002).

Among the taxonomically diverse group of diazotrophs, cyanobacteria are unique because they combine the incompatible processes of oxygenic photosynthesis and the oxygen-sensitive nitrogen fixation within the same organism, sometimes within the same cell. Different groups of cyanobacteria use different strategies for protecting nitrogenase from photosynthetically-generated O_2. Both temporal and spatial segregations of photosynthesis and nitrogen fixation have been characterized in various kinds of cyanobacteria.

In some non-heterocystous cyanobacteria such as *Plectonema*, *Phormidium* and *Pseudoanabaena*, nitrogen fixation and photosynthesis are temporally separated. Genes required for photosynthesis and N_2-fixation are transcribed at different times. Microaerobic condition (up to 1.5% oxygen in darkness and 0.5% in light) is required for the nitrogen fixation of those organisms, because oxygenic respiration is a critical source of ATP and reductant (Berman-Frank et al. 2003). Similar temporal segregation of photosynthesis and nitrogen fixation has been observed in some other non-heterocystous cyanobacteria, including *Lyngbya*, *Osillatoria* and in unicellular forms such as *Gloeothece* and *Cyanothece*. A microaerobic atmosphere does not limit nitrogen fixation in these species (Gallon 2001, Berman-Frank et al. 2003). In light:dark cycles, high nitrogenase activity occurs during times of high rates of respiration, but not during times of peak photosynthesis. Cyclic photophosphorylation at the end of the light period is considered to be an efficient source of ATP, supporting a high rate of nitrogen fixation at the

beginning of the dark period when photosynthesis has stopped and respiration has consumed most oxygen in cells (Tucker et al. 2001). Circadian control of nitrogen fixation was observed in *Cyanothece* when cells were grown in continuous light or darkness (Colon-Lopez et al. 1997, Schneegurt et al. 2000).

Trichodesmium is distinguished from other non-heterocystous cyanobacteria in that it fixes nitrogen only during the daytime. It has a unique strategy for nitrogen fixation, in which photosynthesis and nitrogenase activity are separated both spatially and temporally (Berman-Frank et al. 2001). Nitrogenase occurs in a portion (typically between 10 and 20 percent) of the cells that are often arranged consecutively along the trichome. Spatial segregation exists, but is not sufficient to protect all nitrogenase from O_2 damage since photosynthetic activities also occur in those cells. Expressions of nitrogenase genes and photosynthetic genes are controlled by a circadian clock, with a temporal phase difference of ~6 hr. The result of this temporal segregation is that high nitrogen fixation occurs at a time during the light period when there is a net negative production of oxygen (Berman-Frank et al. 2003).

In contrast to non-heterocystous cyanobacteria, some filamentous genera, such as *Nostoc* and *Anabaena*, have evolved a specialized kind of cell, called a heterocyst (or heterocyte), that is irreversibly differentiated from vegetative cells, and that is specialized for nitrogen fixation (Böhme 1998, Adams and Duggan 1999, Adams 2000). Heterocysts have thick cell walls which are nearly impervious to oxygen. They also contain unpaired thylakoids that are incapable

of oxygen evolution. Energy that is required for N_2-fixation is supplied by cyclic photophosphorylation and reductant is provided by adjacent vegetative cells through intercellular junctions. Organic nitrogen is then supplied from heterocysts to vegetative cells through the same junctions. These features allow nitrogen fixation in heterocystous cyanobacteria to be spatially separated from oxygenic photosynthesis (Adams 2000, Berman-Frank et al. 2001).

As in isolated cultures of cyanobacteria, nitrogenase activity of microbial mats is related to energy availability and oxygen concentration (Villbrandt and Krumbein 1990, Steunou et al. 2008). In early stage of development of some mats, nitrogenase activity was observed to occur almost entirely in darkness. These mats did not turn anoxic during the night and oxygenic respiration provided ATP for nitrogen fixation. Some well-developed mats, demonstrated a small sunset peak and a large sunrise peak in nitrogenase activity, with very low sustained rates of nighttime nitrogen fixation. It was hypothesized that light intensity, photosynthetic activity and oxygen concentrations in mats are low during peak times of nitrogen fixation activity, but are sufficient to supply energy needs of nitrogenase. In contrast to undeveloped mats, these mature mats probably turned anoxic during the night, so the concentration of O_2 retards respiration at night, thus limiting the availability of energy for nighttime nitrogen fixation (Villbrandt and Krumbein 1990, Steunou et al. 2008). Some conceptual models regarding nitrogen fixation of microbial mats have been built based on

measured nitrogenase activities with respect to energy and oxygen (Villbrandt and Krumbein 1990, Steunou et al. 2008).

Previous research has demonstrated that nitrogen fixation in microbial mats results from a combination of activities of various kinds of microorganisms within the mat community. However, the magnitude of these contributions by individual organisms has not been characterized. This study is a characterization of nitrogen fixation within microbial mats from the South Texas coast, focusing on the contribution of a single species. The results of this study demonstrate that a single species of diazotrophic cyanobacterium is responsible for most nitrogenase activity in these mats. Nitrogen-fixing characteristics of this organism (*Hydrocoleum sp*.) are very similar in isolated cultures and in intact mats. This study also provides an insight into how these mats develop and remain viable during droughts.

CHAPTER TWO: MATERIALS AND METHODS

A. Collection and maintenance of mats

Intertidal microbial mats were collected from a sandy beach in a channel connecting the open Gulf of Mexico with Corpus Christi Bay, at a site near Port Aransas, Texas (N 27°51.765' W 97°4.796'). Samples were collected during a 4-year period, starting in 2007, and were obtained at least once during every season except during long drought periods when the mat structure became fragile and less well organized. Rectangular sections of mats greater than 10 cm in each horizontal dimension were collected by cutting down at least 1.5 cm below the surface, lifting the intact structure from the sand, and placing it into a plastic container. Mat sections were submerged in local seawater (~30 ‰ salinity) and maintained in darkness for transport back to the laboratory. Samples were maintained by placing the plastic container in a greenhouse within 9 hours after the time of collection, where they were kept covered with clear Cellophane® and exposed to natural diel sunlight. Mats that were submerged at the time of collection were kept submerged while those exposed to air at the time of collection were not submerged during their maintenance, but were kept wet with seawater from the same area. The temperature in the greenhouse ranged from approximately 15 to 35˚C.

B. Single strain isolation and maintenance

1. Single strain isolation

Individual filaments were separated from the mat by first placing a small piece of the upper layer into a 20-ml glass centrifuge tube, and then inserting the bottom of the test tube into a sonic bath at room temperature until multiple single filaments were separated from the larger piece. Single filaments from the sonicated sample were picked up under a stereo microscope with modified Pasteur glass pipet (Stein 1973), and then transferred into separate Petri plates containing fresh ERD medium (2.3 mM $NaNO_3$, 0.67 mM Na_2HPO_4, 1 ml Vitamin B_{12} and 12 ml PIV metal solution in 1L of seawater). Petri plates were put in darkness for at least 12 hours before illumination (70 – 80 $\mu E/m^2 \bullet s$, 12h light:12h dark) at 25 ˚C. Growth was visible on the surface of Petri plates after several weeks. Cultures on plates that showed only one cyanobacterial morphology were transferred to test tubes and/or flasks in ERD medium for maintenance. Cyanobacteria of three morphologies, originally designated as MF1, MF2 and MF3 could be isolated by this method. A prominent cyanobacterium component of all mature mats, identified as *Microcoleus*, could not be isolated in ERD medium.

2. Single strain maintenance

All three isolated strains of cyanobacteria were kept in liquid ERD medium in 250-ml flasks. The incubation was at 25 °C with diel illumination (12hr light :12hr dark). Cultures were transferred approximately once per month. Strains were

cryopreserved for long-term storage. Cultures could be cryopreserved with high viability when cooled at rate of approximately 1 ˚C/min to -50 ˚C, then placed in a liquid nitrogen storage dewar maintained at -190 ˚C. Cultures were cryopreserved in ERD medium containing either DMSO (5%, 10%), methanol (5%, 10%) or glycerol (10%, 20%). Cultures were thawed as rapidly by placing cryovails into a warm (36 ˚C) water bath immediately after removing them from the liquid nitrogen dewar. Thawed strains were kept in darkness for at least 12 hr before being placed in the normal culturing conditions to facilitate resumed growth. The most effective cyroprotective agent was found to be 10% methanol.

C. Microscopic observations

1. Light microscopy

Small pieces of mat were taken from various locations on collected samples with forceps, placed on glass slides and observed using bright-field and DIC Nomarski optics with an Olympus BX-60 compound optical microscope equipped with an Olympus DP70 digital camera. Filaments from unialgal cultures were observed using this same method.

2. Scanning electron microscopy

Samples from a unialgal culture of MF1 (identified as a *Hydrocoleum* species) were prepared for scanning electron microscopy. Filaments were fixed in a solution containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer. A Pelco BioWave® Pro equipped with vacuum chamber and

ColdSpot® (Ted Pella, Inc. Redding CA. USA) cold plate was used for fixation at 150 W for 1 min on, 3 min off, 1 min on, and then 650 W for 10 seconds on, 20 seconds off, 10 seconds on. Samples were then rinsed (3x for 10 minutes) with 0.1M sodium cacodylate buffer. Postfix staining and initial dehydration steps were combined, using 4% (w/v) osmium tetroxide and 4% (w/v) ferrocyanide in 50% (v/v) ethanol in a Pelco BioWave® Pro with ColdSpot® at 250W for 40 sec each. Subsequent dehydration was performed in the Pelco Biowave® with successive solutions of 70%, 90% and 100% (2X) ethanol. Dehydrated samples were critical-point dried in a Tousimis Samdri 790 CPD (Tousimis Research Corp., Rockville, MD), placed on aluminum specimen stubs (Ted Pella, Inc. Redding CA. USA) and coated with 20 nm of Platinum Palladium in a Cressington 208 Benchtop Sputter Coater (Cressington Scientific Instruments Ltd. UK). Images were captured from a Zeiss Supra 40 VP Scanning Electron Microscope (Carl Zeiss NTS GmbH, Germany).

D. Light penetration measurement in mature mats

Mature mat samples were cut horizontally into 0.1 mm layers, using a Microm HM 550 Microtome Cryostat. Each layer was attached to a clean glass slide. Slides with samples were put in the spectrometer and the optical density was measured at 665 nm (scanning optical density measurements showed that each layer had essentially the same absorption spectrum from 300 nm to 800 nm).

The optical density values were converted into percent transmittance (T%) and the light attenuation after light passing through each layer was calculated.

E. Acetylene reduction assays of nitrogenase activity

1. Mats

Nitrogenase activities of mat samples were measured by acetylene reduction assays as previously described (Li et al. 2010) (Fig M1). A mat was cut vertically into square pieces (~0.5 cm X 0.5 cm), and each piece was placed into a separate 25 ml glass reaction vessel. For samples that were originally submerged at the time of collection, ten ml of ERD medium without the NO_3^- component was placed into each vessel, which totally submerged the mat samples. For samples that were exposed at the time of collection, no medium was added. The colored upper layer of mature mats could be separated from the lower layer with a scalpel, without significantly disturbing either layer, so nitrogenase activity could be determined in separate layers as well as in intact mat samples. Sealed vessels were placed into a 25 °C incubator without agitation. Except when otherwise indicated, vessels were illuminated at an intensity of 900 – 1000 $\mu E/m^2 \bullet s$ and a diel cycle of 12h light:12 dark (250 W halogen lamp). An Agilent 6890N Network gas chromatographic system equipped with an AT-Alumina column (30 m x 0.53 mm, Alltech) and a flame ionization detector was used to measure activity. After each injection of 100 µl gas from a sample flask, the column was held at 80 °C for 5 min. The

temperature was then gradually increased at a rate of 25 °C/min until it reached 180 °C, and then maintained at 180 °C for 5 min before the end of a run. Acetylene reduction data were collected from individual vessels at 3 hour intervals through an entire diel cycle. After each acetylene reduction measurement, the sample was removed from the flask for chlorophyll a determination. Ethylene production was normalized to Chl a content. In addition to duplicate flasks prepared for acetylene reduction measurements, multiple identical additional flasks were prepared. At each time period when ethylene concentration was measured, duplicate additional mat samples were also removed and were quickly frozen by submersion in liquid N_2. They were maintained at -80 ˚C for later DNA, RNA and protein extractions.

2. *Hydrocoleum* cultures

Nitrogenase activities of *Hydrocoleum* cultures were measured in the same way as described for mats, except that 5 ml of ERD medium without the NO_3^- component was placed into each vessel prior to nitrogenase assays, which completely submerged all algae. During experiments, nitrogenase reaction vessels were incubated at 25 °C with an illumination cycle of 70 – 100 $\mu E/m^2 \bullet s$ under a diel cycle of 12h light:12 dark, unless indicated otherwise in individual experiments. Data were also normalized to Chl a content. Duplicates and frozen samples of *Hydrocoleum* cultures were prepared as described above for mat samples.

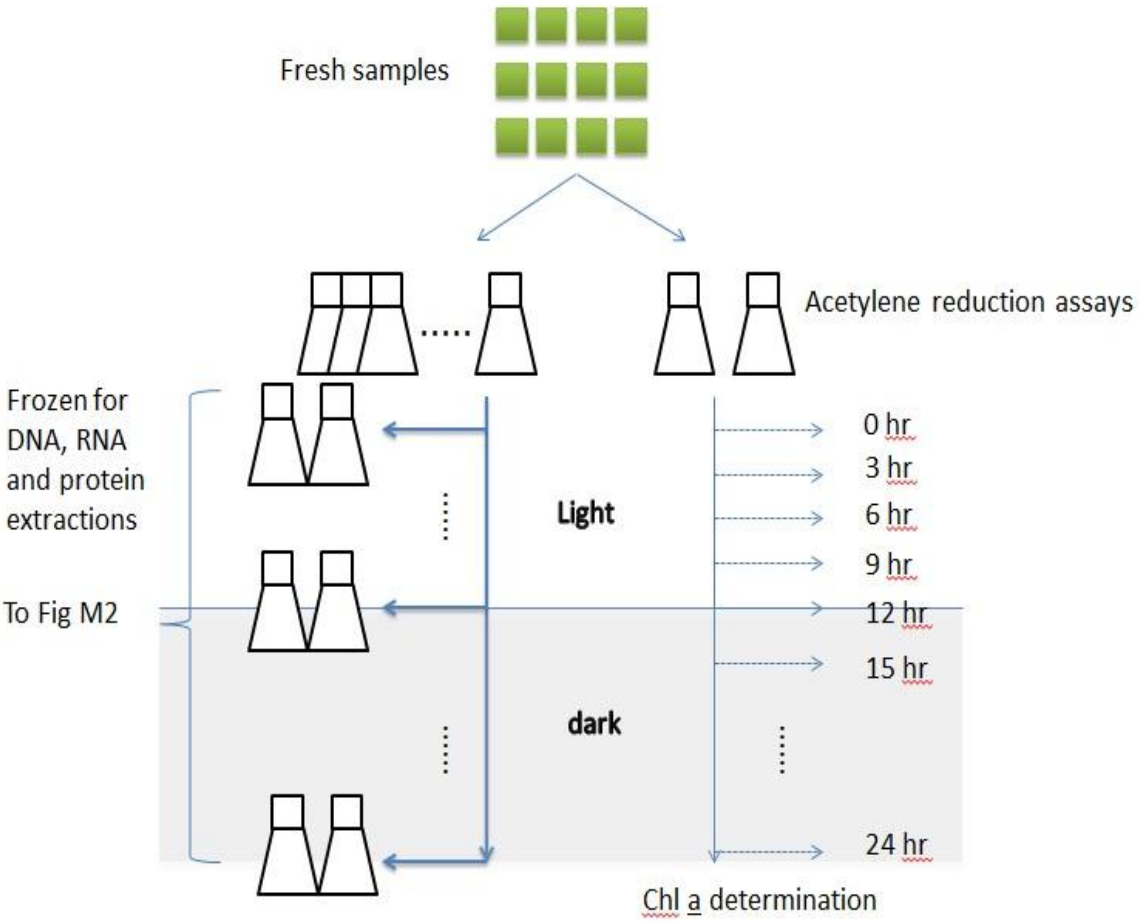


Fig M1. Sample preparation and data collection process in acetylene reduction assays.

F. Total RNA and DNA extraction.

1. RNA extraction

Frozen mat or isolated *Hydrocoleum* samples were homogenized in liquid N_2, suspended in ice cold distilled water, and then separated into two equal volumes. One portion was used for immediate extraction of total RNA (Fig M2 a) while the other portion was stored at -80 °C for later extraction of DNA (Fig M2 b). Total RNA was extracted using a slightly modified Xanthogenate-SDS-phenol protocol (Tillett and Neilan 2000). Homogenized cells were quickly transferred into tubes containing 1.3 ml pre-heated (65 °C) XSP buffer [a 1:1 ratio of phenol to XS buffer (1% potassium ethyl-xanthogenate; 100 mM Tris-HCl, pH 7.4; 20 mM EDTA, pH 8; 1% SDS; 800 mM ammonium acetate)]. Tubes were incubated in 65 °C water bath for 10 min with vortex mixing every 2 min. A 200-µl volume of chloroform:isoamyl alcohol (24:1) was then added to each tube. Tubes were centrifuged for 10 min at 13,000 g and the aqueous phases were transferred to fresh tubes containing 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1). Tubes were incubated at 65 °C for 2 min and then centrifuged at 13000 g for 10 min. Only one extraction with the phenol: chloroform: isoamyl alcohol mixture was performed, in order to minimize the extraction time. The aqueous phases were transferred to fresh tubes and 1 volume of pre-cooled isopropanol was added to each tube. Tubes were inverted several times and set on ice for 15 min to allow the RNA to precipitate. After the final centrifugation (13,000 g for 10 min), the RNA pellets were washed with 70% ethanol, air dried and resuspended in

100 µl RNase-free water. The RNA products, which still contained some carbohydrates and DNA, were further purified using the Roche high pure RNA isolation kit (F. Hoffmann-La Roche Ltd, Basel Switzerland). RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). RNA quality was assessed after the final clean-up step by observations on electrophoretic gels. The sample quality was also evaluated by PCR using *nifH* primers to demonstrate that no *nifH* DNA was present. Purified RNA samples were stored at -80 °C.

2. DNA extraction

As with RNA extractions, total DNA was extracted from duplicate samples that were frozen at the times of GC analyses (Fig M2 b). DNA was extracted using CTAB-SDS buffer according to the method of Li and Brand (Li and Brand 2007). The concentration and quality of DNA were measured with a NanoDrop ND-1000 spectrophotometer, and any necessary clean-up was performed until the A260/A280 and A260/A230 ratios were both above 1.8. Isolated DNA was tested for optimal concentration to be used in real time PCR experiments (see Appendix VI).

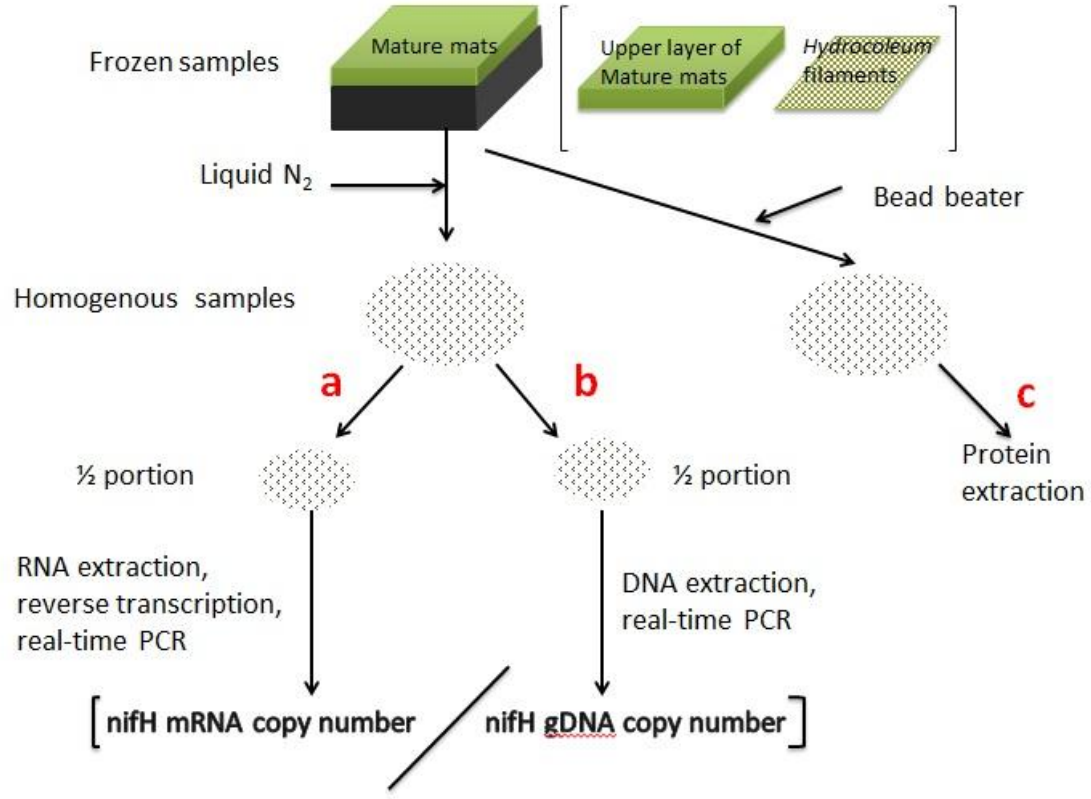


Fig M2. Frozen samples used in RNA, DNA and protein extractions. “a” shows the RNA extraction process, “b” shows the DNA extraction process, and “c” shows the protein extraction process. The same volume of materials from a homogenous sample was used in “a” and “b”.

G. Reverse transcription and real time PCR of the *nifH* gene of *Hydrocoleum*

1. Specific primers design

Hydrocoleum nifH was first amplified with degenerate primers (see Appendix III). The 286 bp PCR product was then purified and ligated into a pCR™4-TOPO® TA vector. The vector was transformed with the TOPO® TA Cloning® Kit (Invitrogen Inc. Carlsbad, CA). Primers that were specific for just the *Hydrocoleum nifH* (forward: 5'-GCTGGTATCAAGTGCGTTGAGT-3'; reverse: 5'-CCACGACCTGCACAACCTACT-3') were designed using primer design tools in IDT (Integrated DNA Technologies) and used in reverse transcription as well as in quantitative real time PCR. To test the specificity of these primers, genomic DNAs from various sources were used individually in combinations as templates in PCR reactions. PCR products were separated on high concentration agarose gels (>1.5%) and then purified for sequencing (see Appendix IV).

2. Reverse transcription

The total RNA from isolated *Hydrocoleum* cultures and from mat samples was diluted before reverse transcription. For best results in final real-time PCR, the optimal concentrations of RNA samples were tested (see Appendix VI). Diluted RNA samples were reverse transcribed using SuperScript® III Reverse Transcriptase (Invitrogen Inc.Carlsbad, CA) kit, with an extension temperature of 50 °C for 60 min.

3. Quantitative real-time PCR

The pCR™4-TOPO® TA vector with the insertion of the target 286 bp *nifH* sequence was used as a standard in real-time PCR measurements. The resulting cDNA from reverse transcription and diluted genomic DNA were amplified by quantitative real time PCR (ABI 7900HT, Applied Biosystems, Carlsbad, CA) in order to determine the original copy number of *nifH* transcripts and the gene copy in the extracted samples. A FAM-5' labeled probe (5'-TGGTGGTCCTGAGCC-3') and Taqman® Universal PCR Master Mixture (ABI) was used during PCR with the protocol of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec, and 60 °C for 45 sec. Triplicate 10 µl reaction mixtures, each containing 2 µl of sample, were used for amplification. A serial dilution of the plasmid carrying *Hydrocoleum nifH* was prepared and the plasmid dilutions were amplified at the same time as samples. The absolute *nifH* gene copy number and the *nifH* transcript number from each sample was determined from a standard curve (see Appendix VI). The cDNA copy number from Fig M1. "a" passway was normalized to the *nifH* gene copy number from Fig M1. "b" passway. The final data were shown as the expression level *of nifH* per gene dose.

H. Protein extraction and western blotting.

1. Protein extraction

Total protein was extracted from frozen mats and from frozen *Hydrocoleum* cultures according to Steunou et al (Steunou et al. 2008). Samples were placed into 2 ml screw-cap tubes with 250 µl ice-cold buffer (50mM Tris-HCl, 50 mM EDTA, 5 mM NaCl), 20 µl DTT (100 mM) , 5 µl of protease inhibitor cocktail (Calbiochem, La Jolla, CA, USA) and ~120 mg glass beads. Tubes were placed in a pre-cooled holder in a mini-beadbeater (GlenMills Inc.). Disruptions of samples were performed by four consecutive runs of 40 sec each. The lysates were centrifuged at 11,800 g for 5 min at 4 °C. The pellets were placed into 200 µl of Na_2CO_3 (0.05 M), followed by the addition of 200 µl of sample buffer (5% SDS, 10 mM EDTA, 20% sucrose) and brought into suspension by vortexing and sonication. No DTT was added to the sample at this time (in contrast to the procedure of Steunou et al.) in order to obtain meaningful protein concentration measurements. Samples were boiled for 5 min and then centrifuged at 11,800 g for 5 min at room temperature. The supernatants were transferred to clean tubes, and concentrations of extracted proteins were determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Twenty µl of sample, 100 µl reagent A' (samples contain the detergent SDS) and 1.0 ml reagent B were combined in a clean glass tube. The tube was vortexed and set at room temperature for 15 min. The absorption value at 750 nm was measured and converted to concentration of

protein according to a standard curve using series dilutions of bovine serum albumin. Protein samples were stored at -80°C for further use.

2. SDS-PAGE and western blotting

Exactly the same total amount of protein (as determined by the Bio-Rad DC assay) was used for each series of measurements (e.g. for mat samples or *Hydrocoleum* cultures at various times during a diel cycle). Samples were loaded into a 10% SDS-PAGE gel (32:1 = acrylamide:bisacryolamide). The gel was run at 120 V for 2 hr and then proteins were transferred onto a nitrocellulose membrane using a Hoefer TE 22 unit. The membrane was incubated for 2 hr with gentle agitation in a blocking solution containing 10 mM PBS and 3% gelatin, and then was washed twice for 15 min each in PBS buffer containing 0.05% Tween-20. Fe-protein antibodies were provided by Dr. Agneta Novéu. The membrane was incubated with the primary antibody (diluted 1:200 in PBS with 1% gelatin) for 2 hr at room temperature or overnight at 4 °C, and then was washed twice in the PBS-Tween buffer for 15 min each. The membrane was incubated with a 1:1000 dilution of HRP-conjugated secondary antibody (BioRad Cat. # 172-1019) for 1 - 2 hr at room temperature, followed by two washes in the PBS-Tween buffer, after which it was ready for staining. The stain solution contained 0.1% (w/v) DAB (3-3'-diaminobenzidine tetrachloride) and 0.3% (v/v) H_2O_2 in PBS solution. Bands which indicated the appearance of Fe-protein were visible after a few seconds.

I. Phylogenetic analyses of isolated cyanobacterial strains.

16s rDNA partial sequences of isolated strains were amplified with the primers of Svenning et al (Svenning et al. 2005). PCR products were cloned with the TOPO® TA Cloning® Kit and sequenced. The sequence of each cyanobacterial strain was compared to existing sequences in GenBank (NCBI at NIH) and aligned with similar sequences using ClustalW. An unrooted tree for the *Hydrocoleum* culture was constructed using Phylip 3.67. Bootstrap values were obtained for both Maximum likelihood (ML) and Neighbor joining (NJ) analyses. Unrooted trees were determined for MF2 and MF3 as well. The phylogeny of the *Hydrocoleum* culture based on its *nifH* sequence has also been analyzed using the same analysis.

J. Chlorophyll a determination for mats and *Hydrocoleum* cultures

Chlorophyll a was extracted from mature mats, proto-mats and *Hydrocoleum* cultures with 100% methanol as follows. Samples were first separated from any liquid and blotted to minimize adhering liquid. Each sample was put into a 10 ml test tube and 5.0 ml of pure methanol was added to the tube. A spatula was used to disrupt relatively firm intact mat material. Tubes were left at room temperature for 10 min in darkness and then centrifuged at 3500 rpm for 10 min. The clear supernatant in each tube was transferred to a cuvette and the absorption value at 665 nm was measured using a Shimadzu UV-VIS spectrophotometer (Shimadzu Group, Kyoto, Japan). The exact volume of the supernatant was determined. The

extraction solution could be kept overnight at -20 °C without significant changes in the absorption value. The concentration of Chl a and the total amount of Chl a in each sample were calculated using an extinction coefficient of 12.955 (Ritchie 2006).

K. Cell number count for filamentous cyanobacteria

The method described here is most reliable if cells within filaments are uniform in length, as is true for *Hydrocoleum* (Fig 19). The long, aggregated filaments in *Hydrocoleum* cultures were first homogenized with a glass homogenizer equipped with a Teflon® pestle. Cells were not damaged during the process, but aggregates were disrupted and filaments were fragmented. Homogenized cultures were diluted with fresh ERD medium to allow counting. Cell counts were performed by using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA) and a light microscope as follows. The total length of filaments seen in a 1 mm X 1 mm square area under the microscope was measured and converted to the number of cells based on the average length of each cell. For each sample, counting was repeated 5 times to get an average cell number in a 0.1 mm^3 volume.

L. Measurement of growth rate of *Hydrocoleum*

Hydrocoleum doesn't grow in homogenous liquid culture. Filaments begin to aggregate and attach to the surface of a glass container almost immediately after

inoculation. Its approximate doubling time was estimated by measuring the Chl a concentration at various times. A linear relationship between the number of cells and concentration of extracted Chl a was established using the method described above. Eight identical 10-ml test tubes were prepared and 5 ml of homogenized *Hydrocoleum* cultures was transferred into each tube. Cell concentrations were sufficiently high that meaningful Chl a measurements could be determined soon after inoculation, but not so high that the aggregating filaments precluded exponential growth. All test tubes were maintained under the same culture conditions. At 24 hr-intervals, the *Hydrocoleum* from two tubes was collected by centrifugation and then extracted with methanol for Chl a measurement. The approximate doubling time over a 4-day interval was determined from these measurements.

CHAPTER THREE: RESULTS

I. NITROGEN FIXATION IN MICROBIAL MATS FROM SOUTH TEXAS

A. Collection and microscopic observations of microbial mats

The collection site is shown in Fig 1. Mats occurred on a sandy beach along a shallow lagoon. The area was frequently disturbed by vehicle and human foot traffic. Mats were studied primarily from relatively undisturbed sites, but disturbed areas were recolonized by mats in wet weather. The appearance of the site changes significantly with surface moisture. Some beach areas are submerged in water during wet seasons. Mats were frequently observed in shallow (less than 10 cm deep) pools as a green or yellow-green surface on the sand (Fig 2a). Similar mats occurred in areas not covered by water providing that the surface was near-saturated (Fig 2b). Salinity at the collection site varied widely during wet seasons, depending on recent rainfall, but ranged between 15 and 30‰ at collection times. During droughts, the sandy surface became dry down to several mm, with no evidence of any mat on the surface (Fig 2c). However, at many locations, a blue-green layer 1- 2 mm thick was seen several mm below the surface. Various morphologies of mats or mat-like structures found in the same collection site are shown in Fig 3.

Fig 1. Mat collection site. This map shows an area of South Texas Coast near Port Aransas. Red arrow indicates the location of the collection site.

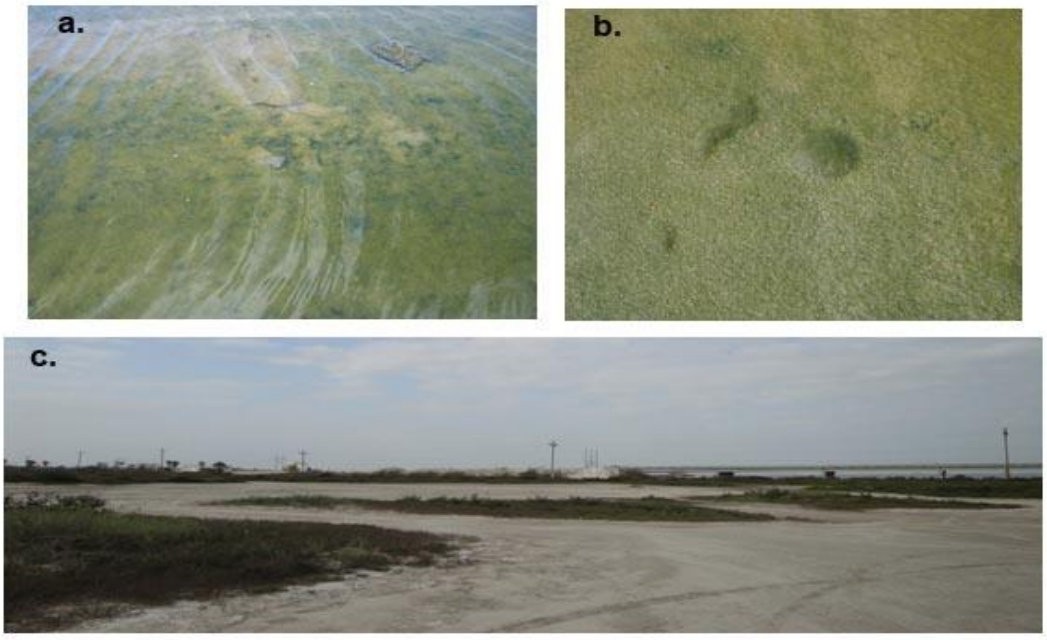


Fig 2. Appearance of the mat collection site under different climactic conditions. a. wet season: green/yellow-green surface, submerged in water. b. wet season: green/yellow-green surface exposed to air but near saturated. c. dry season: sandy surface; no mats visible.

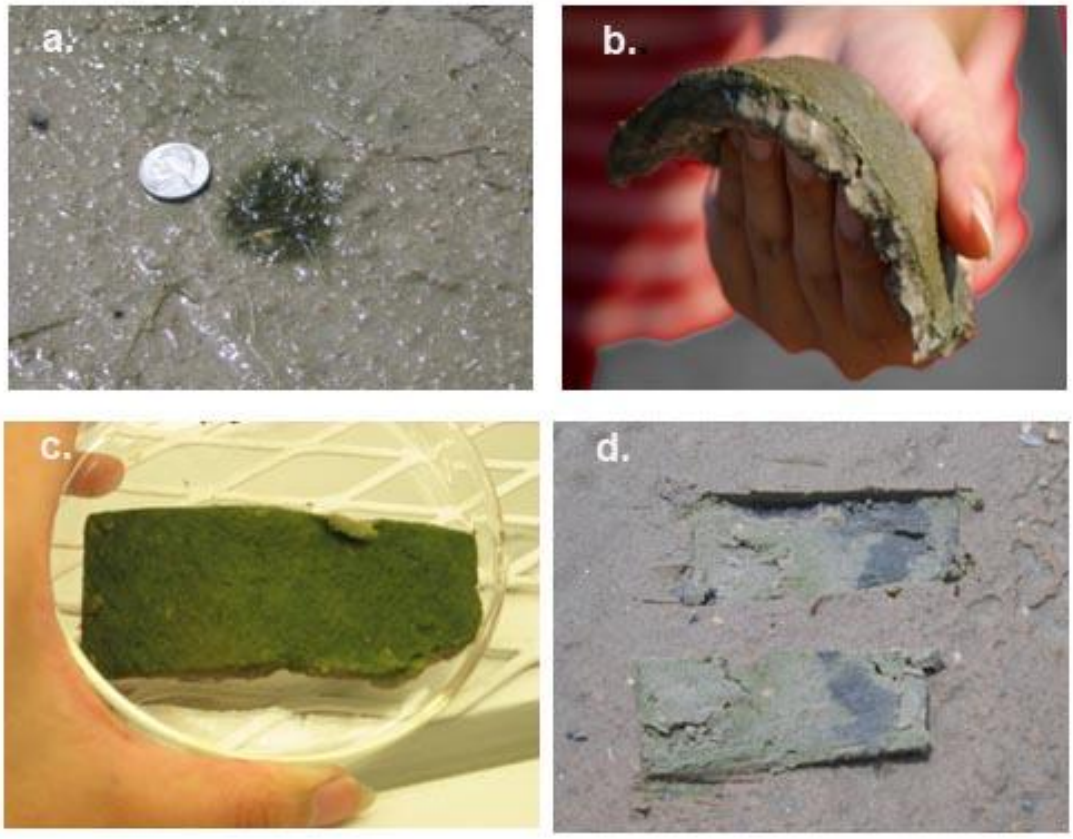


Fig 3. Various morphologies of mats or mat-like structures at the collection site. a. A small surface-exposed circular green patch observed in a water-saturated area in a wet season. b. A section cut from a submerged mat in a wet season. c. A section cut from a surface-exposed mat in a wet season. d. A section cut from the sandy surface in a dry season. The top rectangle shows the area from which the section was removed. The bottom rectangle shows the removed and inverted section. Note the blue-green patch seen in both rectangles.

In wet seasons, sections of mats several hundred cm^2 in area could be removed intact from the surface of the sand. The thickness of these mats was highly variable, ranging from <0.2 to >1.0 cm. The vertical profile revealed two distinct layers (Fig 4). The thickness of the upper green (sometimes yellow-green) layer varied from 0.1 to 0.5 cm and was dominated by non-heterocystous filamentous cyanobacteria, mostly *Microcoleus*-like and *Hydrocoleum*-like cyanobacteria (Fig 3b, c). Thin (typically 2 ~5 µm diameter) cyanobacterial filaments were also seen in the upper layer (Fig 3d, e), but microscopic observations indicated that the two morphologies shown in Fig 3b and c represent over 95% of the cyanobacterial biomass. Although distinct stratification was often observed within the upper colored layer of mats (Fig 3a), the two dominant filamentous cyanobacteria were each distributed throughout the upper layer and no obvious vertical segregation of these two morphologies was seen. The lower layer ranged in color from dark grey or black. It consisted of mostly organic (combustible in a heated crucible) debris (Fig 3f) and sand particles. The very few cyanobacteria which did occur in this layer appeared identical to two dominant morphologies in the upper layer. Heterotrophic bacteria were seen throughout the mat, some of which adhered to surfaces of the cyanobacteria. Mats with this well-developed structure are defined as mature mats.

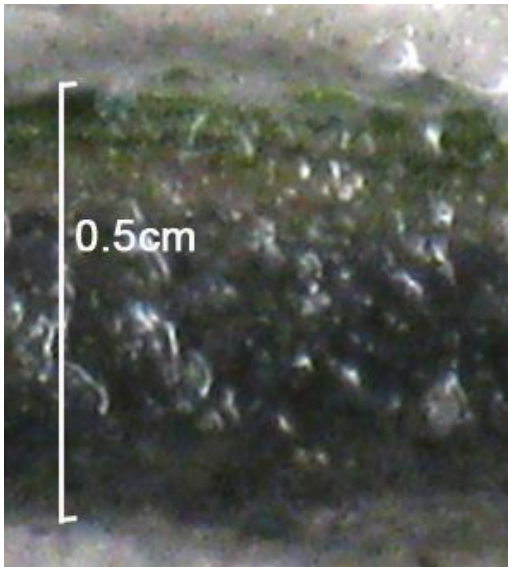


Fig 4. Cross-section of a mature mat. The upper green/blue green layer is ~ 0.1 cm thick; the lower black layer is ~ 0.4 cm thick.

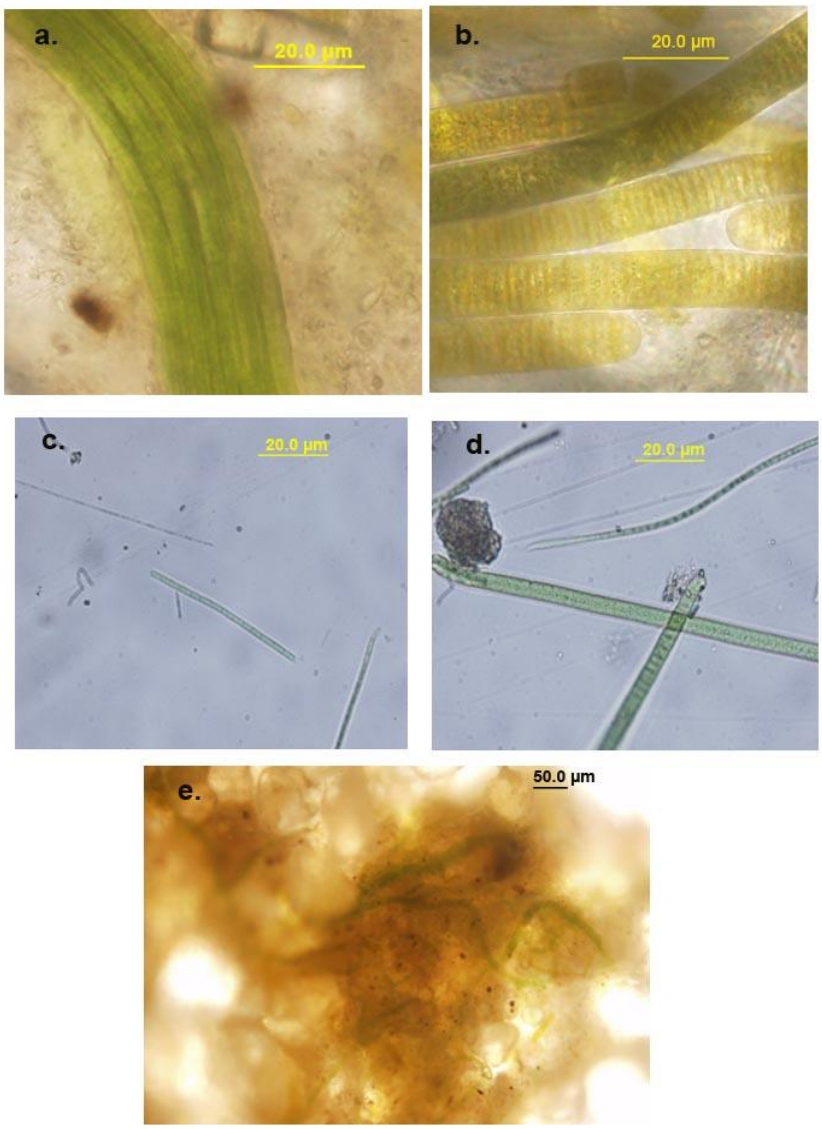


Fig 5. Light microscopic images of cyanobacteria observed in the upper layer of microbial mats and debris in the lower black layer. a. *Microcolues*-like filamentous cyanobacteria concentrated in the upper layer. b. *Hydrocoleum*-like cyanobacteria concentrated in the upper layer. c, d. Thin filamentous cyanobacteria distributed sparsely in the upper layer. e. Aggregated sand grains and debris seen in the lower layer.

The subsurface mat-like structures were collected during drought seasons (Fig 6). No well-defined layer occurred below the blue-green layer, although 1 ~ 3 mm of sand below the blue-green layer adhered weakly. This entire subsurface unit, herein called a subsurface proto-mat, appeared to be structured, but was fragile and did not adhere together when disturbed. The only cyanobacterium observed in the blue-green layer was a *Microcoleus*-like strain (Fig 7a) that appeared identical morphologically to the strain illustrated in Fig 5a. Sand particles below the green layer were held together by a translucent glue-like material (Fig 7b). This material appears identical to the mucilage matrix outside the *Microcoleus* bundles of filaments (Fig 7a).

Fig 6. Cross-section of a subsurface proto-mat. A colored sandy surface layer covers the ~ 0.1 cm thick blue-green layer. A thin layer of loosely-adhering sand particles are attached to the bottom surface of the blue-green layer.

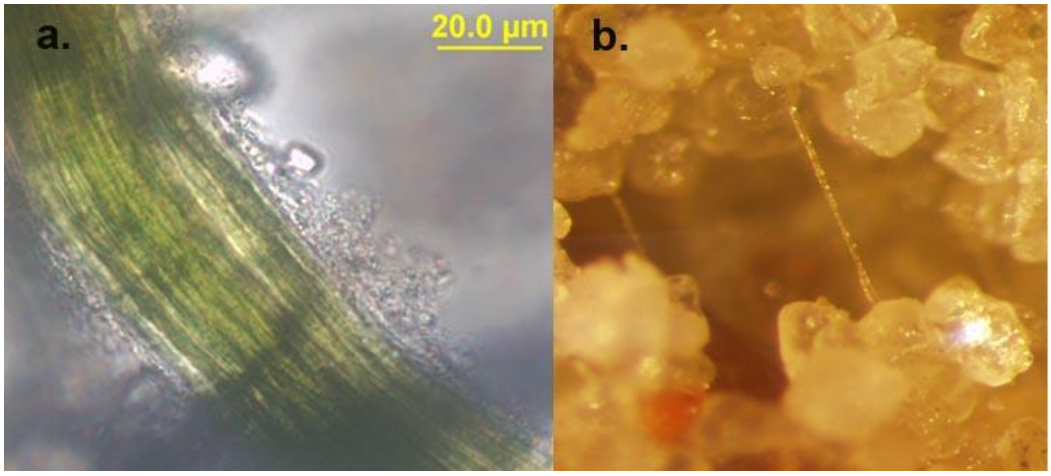


Fig 7. Components of subsurface proto-mats. a. Light microscopic image of the *Microcoleus*-like cyanobacterium that dominates the upper blue-green layer. b. Stereomicroscopic image of the bottom layer, consisting of sand grains loosely held together by a translucent glue-like matrix.

B. Light penetration in mature mats

Light striking the surface of a mat is attenuated due to colored molecules that absorb the light. As shown in Fig 8, the light intensity is already reduced to less than 10% of its initial value at 0.4 mm depth. The average light intensity exposed to organisms distributed through the upper green layer (~1 mm thickness) of mature mats is approximately 10% of the surface light intensity. The maximum light intensity available in the laboratory was 900 ~ 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$, (approximately 50% of full sun intensity), which is expected to expose the upper green layer of mats to an average light intensity of approximately 150 $\mu E \cdot m^{-2} \cdot s^{-1}$

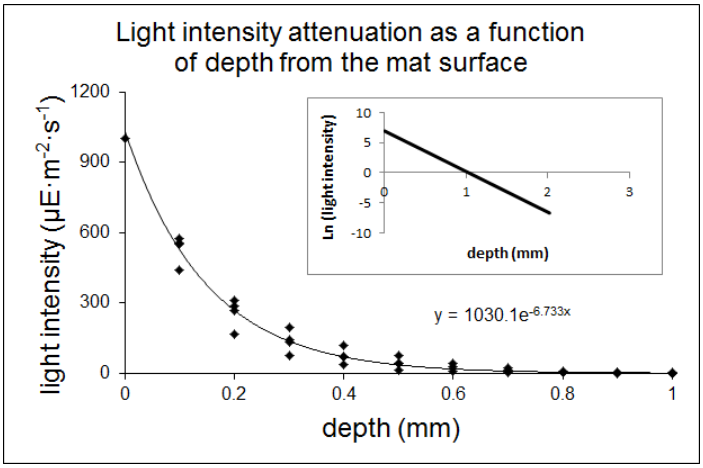


Fig 8. Penetration of light through the top (1 mm thick) layer of a mature mat. Incident light intensity was ~ 1000 μE·m^{-2}·s^{-1}. The light intensity after penetration through successive 0.1-mm horizontal sections was measured. At each depth (0.1 mm between), four replicates of mat samples were measured. A best fit exponential curve ($y=1030 \cdot e^{-6.733x}$) of all data is shown. Points on the graph indicate calculated values based on individual measurements. The inset figure shows the linear relationship between Ln (light intensity) and the depth.

C. Characteristics of nitrogen fixation of mature mats

Fig 9 shows that mature mats can fix nitrogen. Almost all activity occurs in darkness, although a small level of nitrogenase activity (NA) occurs throughout the light period. Activity continues to increase during the entire 12-hr dark period, abruptly stopping upon illumination. The level and pattern of NA in the isolated

upper green layer of mature mats is essentially identical to those features in intact mature mats.

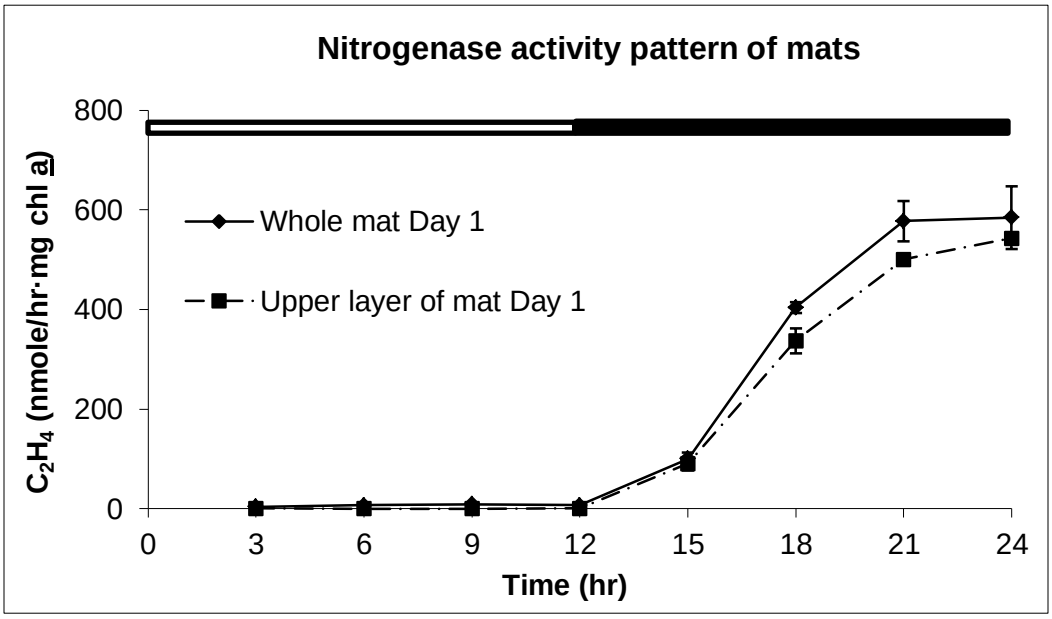
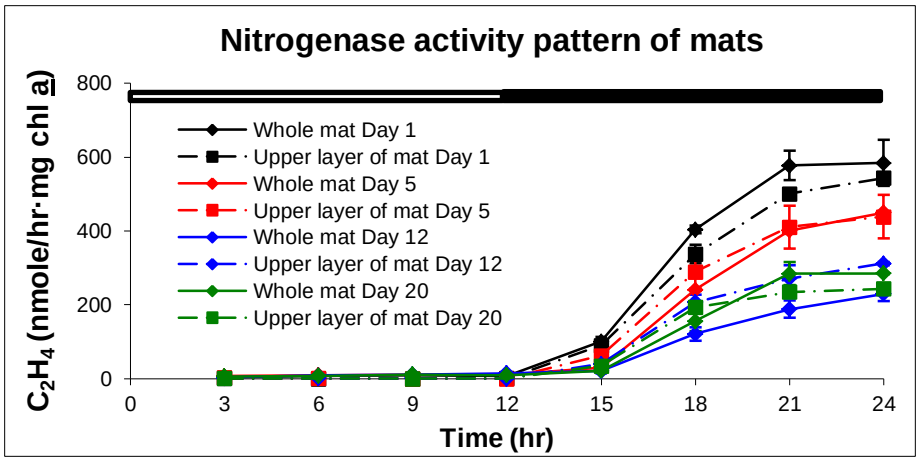


Fig 9. Nitrogenase activities (NA) of intact mature mats and the separated upper layer of mats. The white bar above the plot shows the light period; the black bar shows the dark period. Ethylene production rates are normalized to mg Chl a. Samples were placed in darkness for 12 hrs prior to time zero, so sample conditions at zero-time are the same as sample conditions at the 24-hr time point. Bars show the range of duplicate measurements (n=2).

D. Stability of nitrogenase activity of mature mats

Mats were maintained in the greenhouse under natural sunlight (see Materials and Methods). Nitrogenase activities of whole mature mats and of the isolated upper layer were measured periodically over a 20-day interval. Nitrogenase activity data were recorded at days 1, 5, 12 and 20 after mat collection (Fig 10a). Whole mature mats and the isolated upper layer of mats have the same pattern of nitrogenase activity, which remains unchanged throughout the 20-day period. Rates gradually declined, but peak activity at 20 days was approximately 50% of the peak value in day 1. The data obtained for Fig 10a were recalculated to determine the total amount of N_2 fixed during 12-hr daylight period and the 12-hr night period (Fig 10b). Fig 10b shows that, for each day, C_2H_4 produced in the upper layer alone accounts for more than 90% of total C_2H_4 production in whole mats. Total ethylene production gradually decreased during the 20-day measuring period, but production during the 20^{th} day was approximately 50% of the production during the first day. A small amount of ethylene continued to accumulate during the light period in whole mats, but no ethylene production was observed during the light period in the isolated upper layer (Fig 10b).



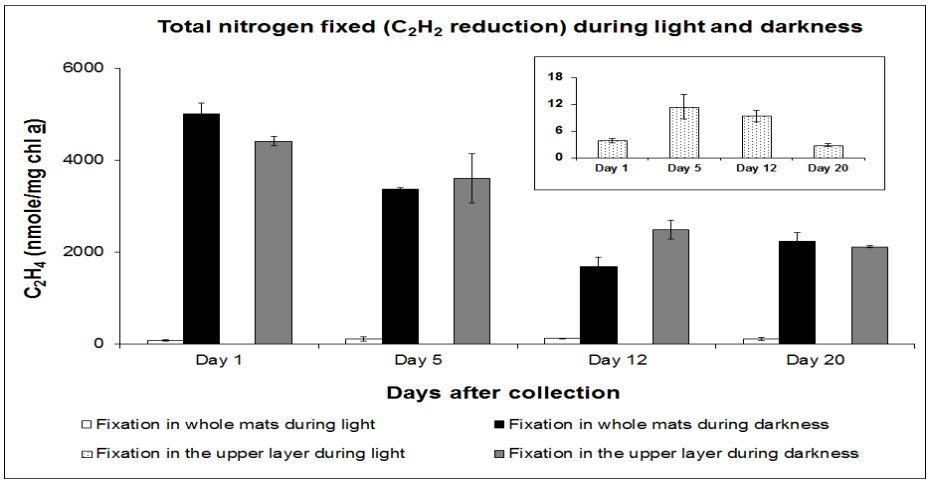


Fig 10. Stability of nitrogen fixation of mature mats and the upper layer of mats during a 20-day period after collection. a. Rates of nitrogenase activity at different time intervals during a diel cycle, measured on several different sampling days. The white bar above the plot shows the light period; the black bar shows the dark period. b. Total amount of ethylene accumulated during daytime and nighttime in each sampling day. Four

bars are indicated for each day shown. Values for measurements of the mat upper layer in light are too low to be visible in the main figure; they are shown in expanded view in the inset. The first sample was measured within 24h after the time of mat collection (Day 1). Bars show the range of duplicate measurements (n=2).

E. Lack of nitrogenase activity in subsurface proto-mats

Mature mats and subsurface proto-mats are morphologically different (compare Figs 4 and 6). Although mature mats had high NA (mostly at nighttime), proto-mats had no detectable NA at any time during the diel cycle. Thus, proto-mats may not have the ability to fix nitrogen (Fig 11).

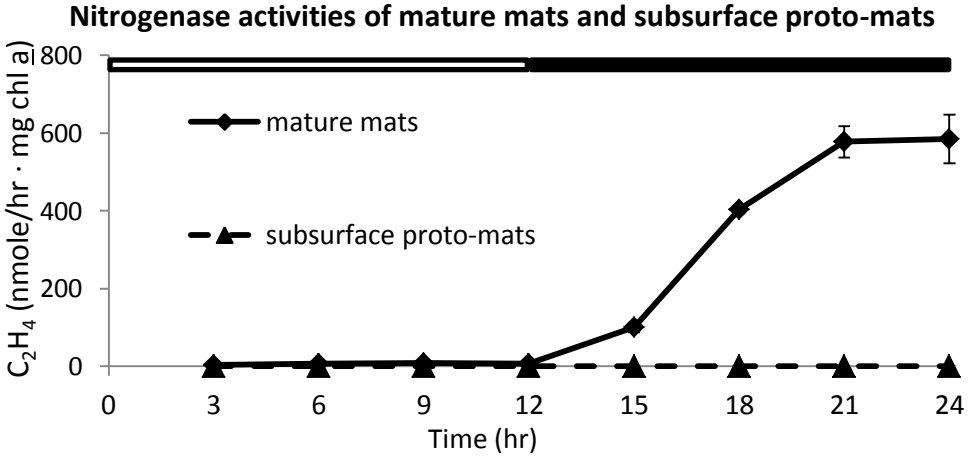


Fig 11. Nitrogenase activities of mature mats and subsurface proto-mats. The white bar above the plot shows the light period; the black bar shows the dark period. Bars (not shown when values were nearly identical) represent the range of duplicate measurements (n=2).

F. Western blotting of nitrogenase reductase (Fe-protein) of mature mats and proto-mats

Total proteins were extracted from mature mats and subsurface proto-mats for western blotting of the Fe-protein (Fig 12). A low level of Fe-protein was observed in mature mats during the first 6 hours of a 12-h illumination period. The concentration increased by the 9th hour in light. It remained high during the remainder of light period and subsequent dark period. In subsurface proto-mats, where no nitrogenase activity could be detected (Fig 11), neither was any Fe-protein detected.

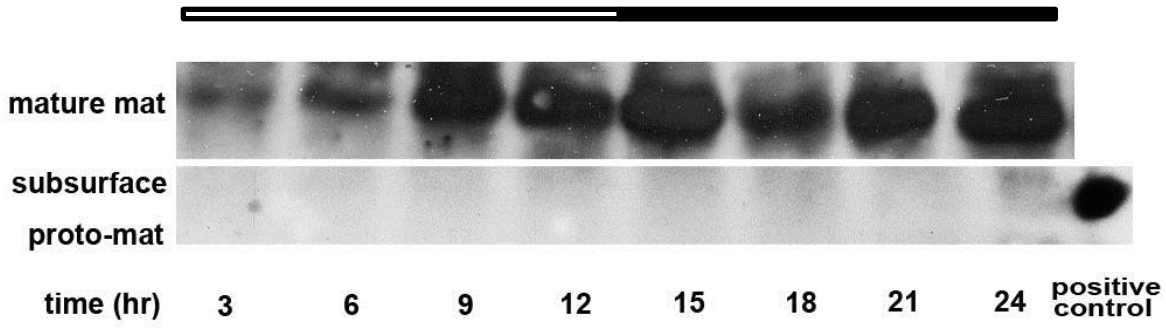


Fig 12. Western blots of Fe-protein in mature mats and subsurface proto-mats. The image is the composite of two sets of overlayed measurements on the figure. The figure was not altered in any other way. The positive control shown with subsurface proto-mat blots is identical to the 12-hr sample of the mature mat. The white bar above the plot shows the light period; the black bar shows the dark period.

G. Nitrogenase activity and Fe-protein expression of an isolated cyanobacterium from mature mats

Several cyanobacteria were isolated as unialgal cultures from the upper layer of mature mats with the method described in Materials and Methods (See Results II). One strain, identified as *Hydrocoleum*, was tested for nitrogenase activity and Fe-protein expression. Acetylene reduction assays were performed as described for mats, except light intensity used in the experiments was 80 ~ 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ instead of 900 ~ 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ for mats. The lower light intensity for measuring nitrogenase activity in isolated cultures was chosen because light is greatly attenuated in passing through the upper layer of intact mats (see Fig 8 and section B). It was estimated that this lower light intensity for isolated cultures was approximately equal to the average intensity to which *Hydrocoleum* was exposed in intact mats that were illuminated at 900 ~ 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$. Fig 13 shows that *Hydrocoleum* is capable of nitrogen fixation, but only at night. The pattern of NA in *Hydrocoleum* cultures is very similar to the pattern in mature mats. Both rates increase during entire dark period, and both quickly decrease to zero at the beginning of the subsequent light period.

Fig 14 shows the pattern of Fe-protein occurrence in isolated *Hydrocoleum* at different times during a diel cycle. A low level of Fe-protein was observed during the first 9 hours of a 12-h illumination period. The concentration then increased toward the end of the illumination period and remained high during the subsequent dark period. A very similar pattern of Fe-protein expression was

observed in mature mat (Fig 12). Protein concentrations were not quantitatively measured, but did not appear to be proportional to the nitrogenase activity (Fig 13 and 14).

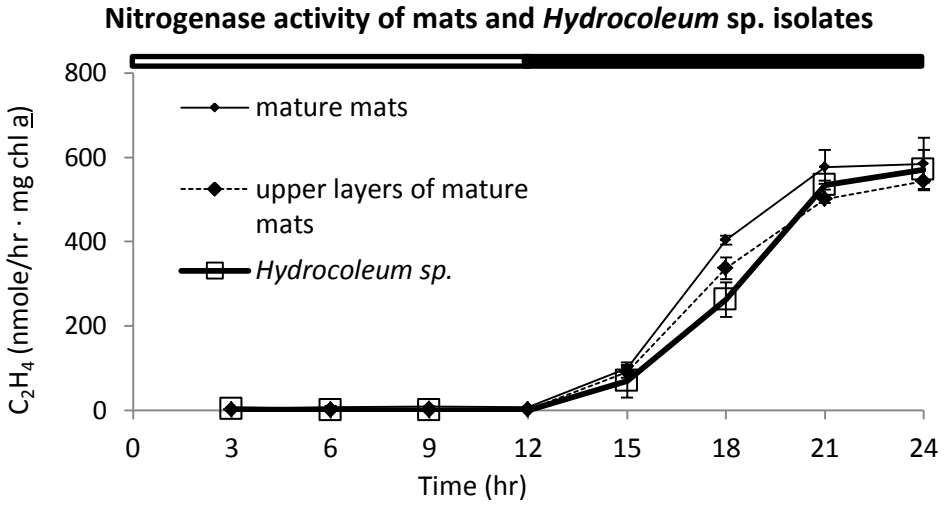
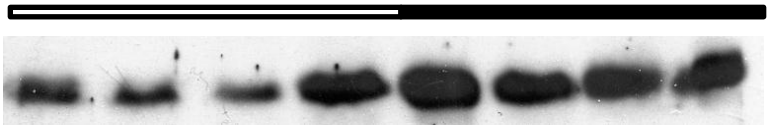


Fig 13. Pattern of nitrogenase activity in an isolated culture of *Hydrocoleum*, compared to the patterns in mature intact mats and in the upper layer of mats. The white bar above the plot shows the light period; the black bar shows the dark period. Bars show the range of duplicate measurements (n=2).



Time (hr) 3 6 9 12 15 18 21 24

Fig 14. Western blots of Fe-protein in isolated *Hydrocoleum* cultures at different times during a diel cycle. The white bar above the plot shows the light period; the black bar shows the dark period.

H. Expression of *Hydrocoleum nifH* in isolated cultures and in mature mats

The levels of *nifH* transcripts in mature mats and in isolated *Hydrocoleum* cultures were determined at various times during a diel cycle (Fig. 15 and Appendix for calculation). The pattern in isolated *Hydrocoleum* cultures was similar to, but somewhat different from, the pattern in mats. The *nifH* mRNA level in mats increased during the entire light period and decreased gradually during the following dark period, with peak expression occurring at the end of the light period. In contrast, the level of expression in isolated *Hydrocoleum* continued to increase for several hours into the dark period, then quickly decreased to near zero. The pattern of expression of *nifH* transcripts in intact mats was virtually identical to the pattern in the separated upper layer (Fig. 15). The *Hydrocoleum nifH* gene was not detected in subsurface proto-mats, consistent with

microscopic observations of no detectable *Hydrocoleum* filaments in subsurface proto-mats.

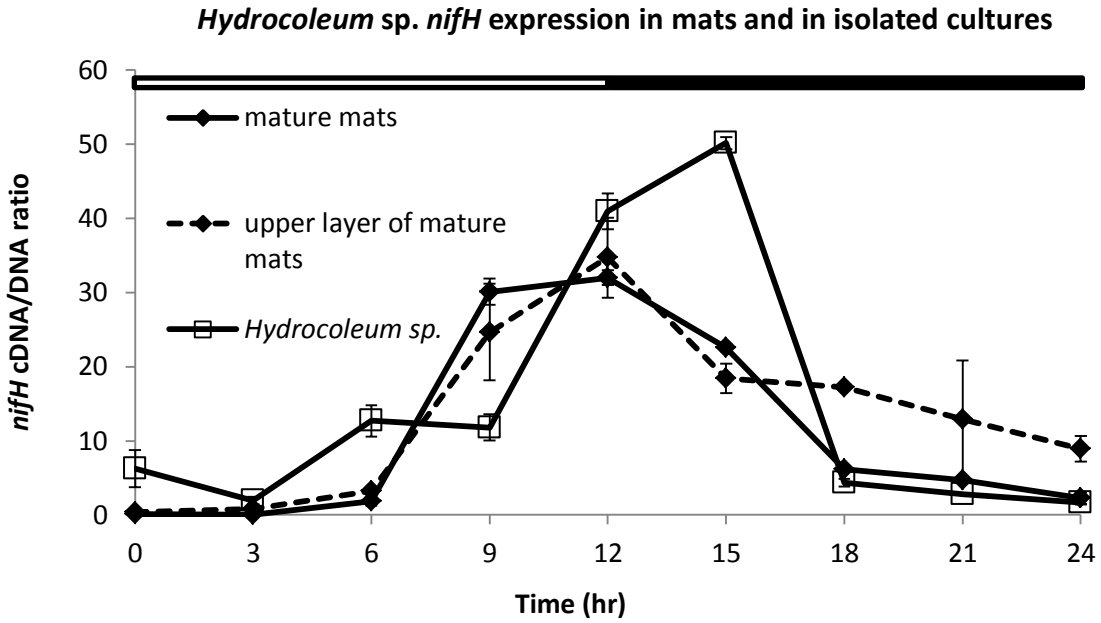


Fig 15. Level of *Hydrocoleum nifH* mRNA in mature mats, in the upper layer of mature mats and in isolated *Hydrocoleum* cultures. The horizontal bar at the top of graph indicates the light period (white bar) and dark period (black bar). Bars show the range of duplicate measurements (n=2).

II. ISOLATION AND CHARACTERIZATION

OF CYANOBACTERIA FROM MICROBIAL MATS

A. Isolation of single strains from mature mats

Three filamentous non-heterocystous cyanobacteria strains were isolated and grown in unialgal cultures. Light microscopic images of these three cyanobacteria are shown in Fig 16. The cyanobacterium shown in Fig 16a, designated as MF1, is one of the two dominant strains found in the upper layer of mature mats (Fig 5b). Diameters of MF1 filaments are ~8 -14 µm. Cells usually have a yellow-green color. The cyanobacteria shown in Figs 16b (designated as MF2) and 16c (designated as MF3) were very sparse in mat samples, but were successfully maintained in unialgal cultures. Cells of these two strains always appeared blue-green in color. Filaments of MF2 were ~ 5 µm in diameter while filaments of MF3 were ~ 2 µm in diameter. Both MF2 and MF3 demonstrated gliding movement.

B. Identification of isolated cyanobacteria strains based on 16s rDNA sequences

Isolated strains of cyanobacteria were identified phylogenetically based on their 16s rDNA sequences. The analysis of MF1, MF2 and MF3 together is shown in Fig 17. Other sequences on the trees are from the NCBI database with the access number showing after the species name. Based on this analysis, MF1

is closely related to *Hydrocoleum* and *Trichodesmium*. MF2 is similar to *Geilterinema*, and MF3 may be a species of *Phormidium* or *Leptolyngbya*. Further analysis for MF1 is shown in Fig 18, with more similar sequences from blast results and information of bootstrap possibilities. MF1 is identified as a *Hydrocoleum* species according to this phylogenetic analysis.

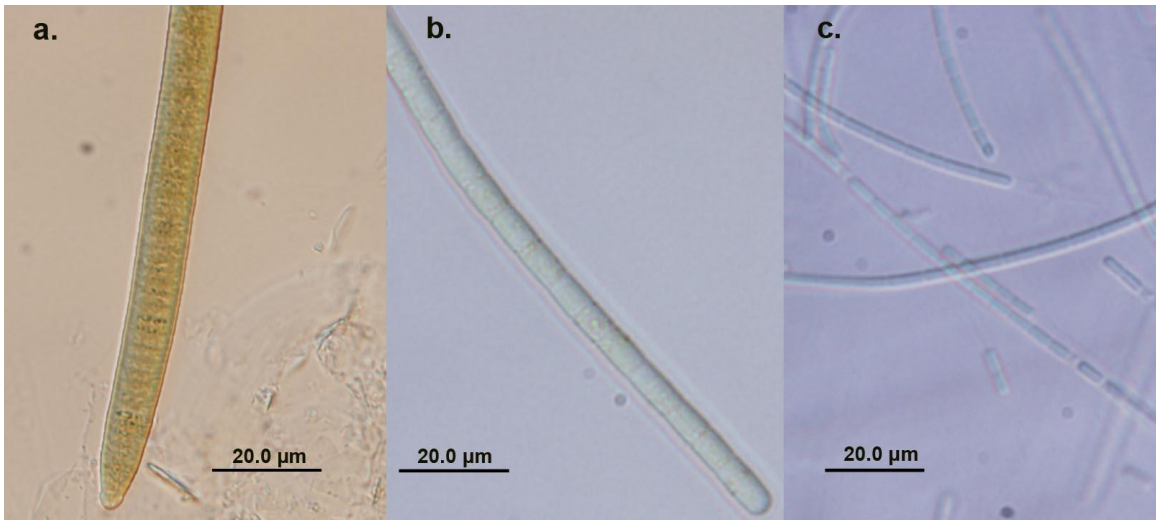


Fig 16. Cyanobacateria isolated from the upper layer of mature mats and cultivated in unialgal culture. a. Bright field microscopic image of MF1. b. Phase contrast microscopic image of MF2. c. Phase contrast microscopic image of MF3.

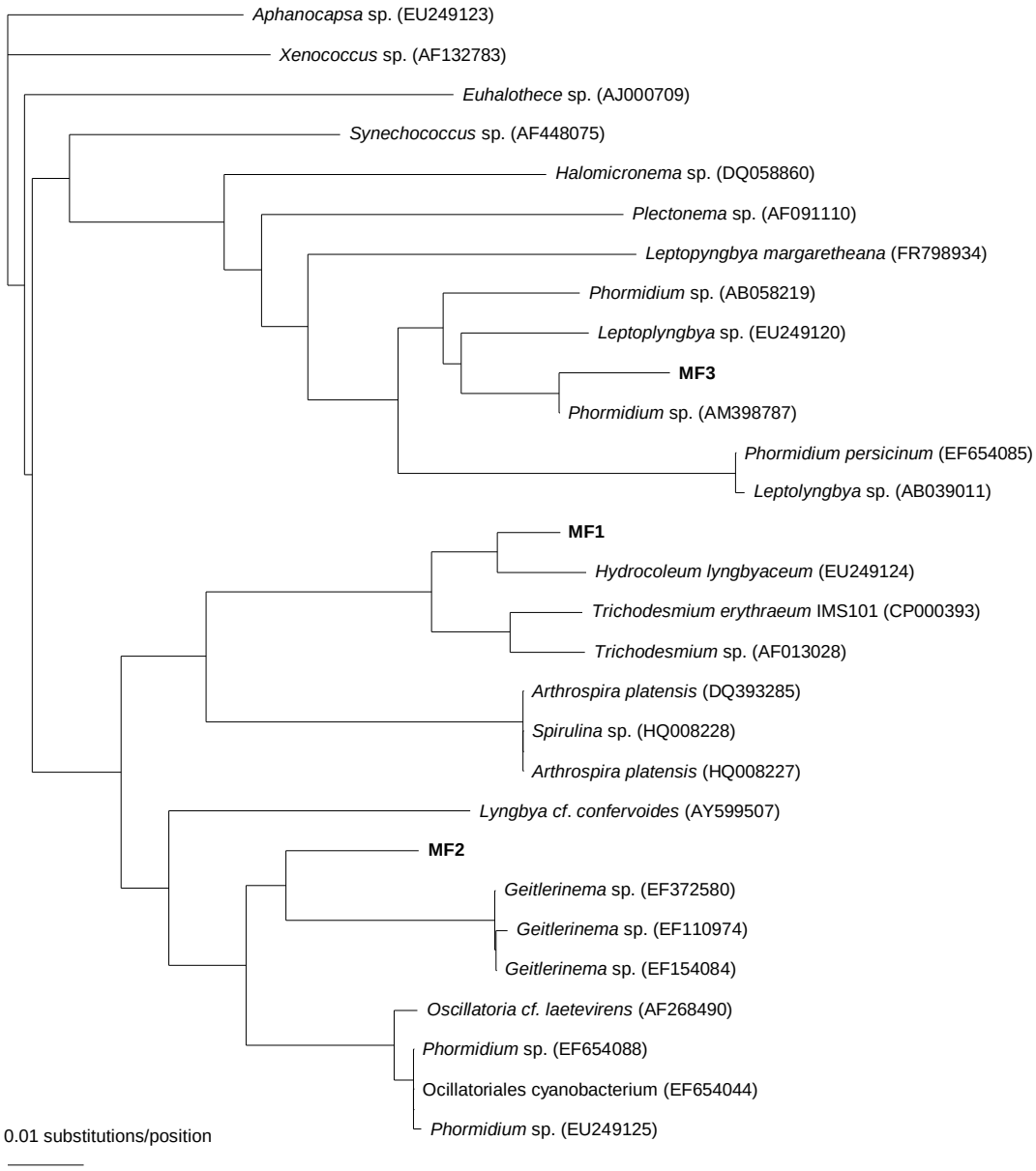


Fig 17. Phylogenetic tree of the isolated MF1, MF2 and MF3, based on16s rDNA. The unrooted tree used the unicellular cyanobacterium *Aphanocapsa* sp. (EU249123) as the outgroup. The tree was constructed using DNAdist

and Neighbor joining. Branch lengths are proportional to the number of substitutions per position.

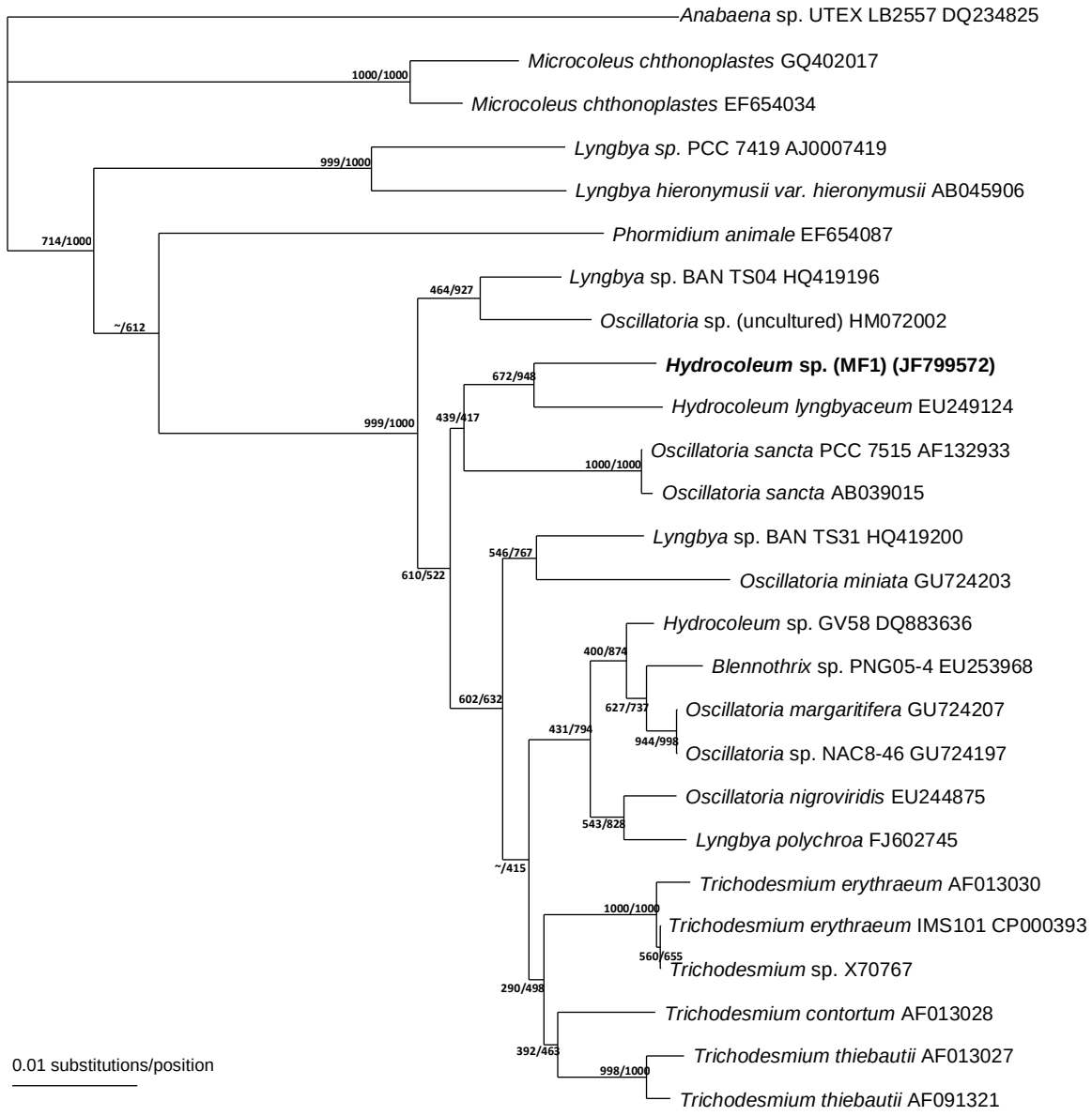


Fig 18. Phylogenetic tree of isolated MF1, based on16s rDNA. The unrooted tree used *Anabaena* sp. (UTEX LB2557) as the outgroup. Numbers at nodes represent bootstrap probabilities of ML (maximum likelihood)/NJ

(neighbor joining) in 1000 replicates. Branch lengths are proportional to the number of substitutions per position.

C. General characteristics of *Hydrocoleum*

1. Doubling time

The growth rate of *Hydrocoleum* was measured as described in Materials and Methods. There is a linear relationship between the cell number and the OD 665 value of the methanol extract (Fig 19a). During the first 3 days of growth after initial inoculation, an approximately consistent growth rate was observed and the doubling time was ~ 42 hr.

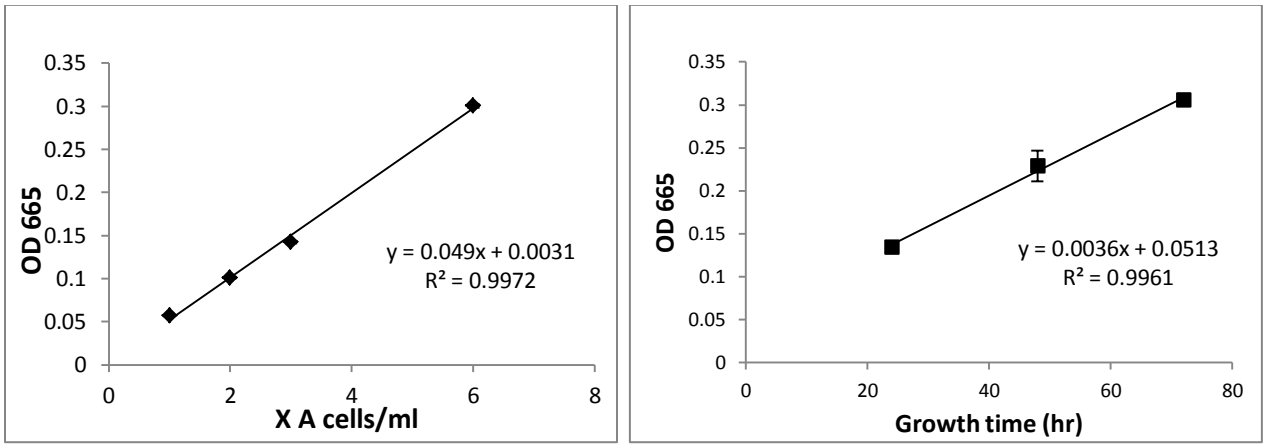


Fig 19. Optical density of *Hydrocoleum* at 665 nm as a function of cell concentration and of time of measurement after initial inoculation. Bars indicate the range of duplicates (n=2).

2. Phase contrast microscopic observations

Hydrocoleum filaments were observed by differential interference contrast (DIC) microscopy. Fig 20 demonstrates that individual trichomes of *Hydrocoleum* are surrounded by a sheath. Trichomes are cylindrical and slightly constricted at cross walls (Fig 20a). The tips of undamaged trichomes are tapered and the cell at each end forms a cap-like structure which is morphologically distinct from intercalary cells (Fig 20b). The distinguishing characteristics of this strain match the standard description of *Hydrocoleum* (Komárek and Hauer, 2011).

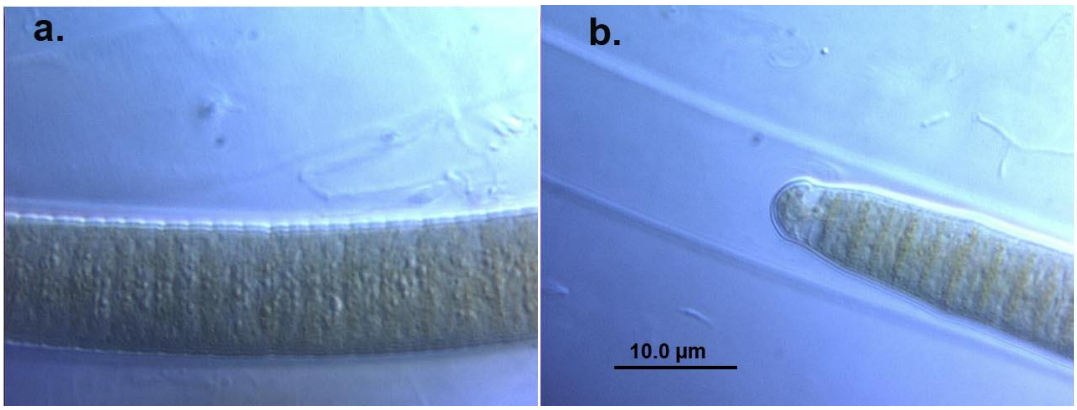


Fig 20. DIC images of Hydrocoleum filaments. a. Central portion of a filament. b. Tip of a filaments.

3. Scanning electron microscopic observations of *Hydrocoleum*

When cultured in undisturbed glass flasks, individual or small aggregates of filaments became attached to a surface of the flask, continuing to replicate into a radiating film, and eventually forming a thick biofilm containing multiple layers of

cyanobacteria. This film remained loosely attached to the flask (Fig 21). Scanning electron micrographs showed that the film consisted of individual filaments held in place by a continuous matrix (Fig. 22a). Sheaths surrounding individual filaments were usually not evident in trichomes embedded within the matrix (Fig. 22b), suggesting that the matrix consists of a confluence of sheath material. Heterotrophic bacteria were seen on the surface of filaments, adhering tightly through a material that appeared similar to sheath material (Fig. 22c).

Fig 21. Biofilm structure formed in a *Hydrocoleum* culture, showing loose attachment to glass surfaces. The arrow shows a portion of the biofilm that was released from a glass surface upon gentle agitation.

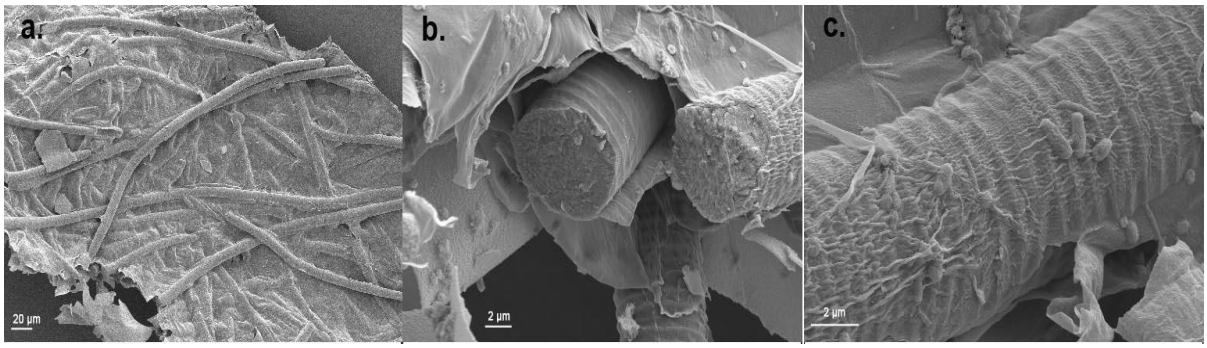


Fig 22. Scanning electron micrographs of isolated *Hydrocoleum* sp. in liquid cultures. a. Surface view of a biofilm, showing filaments held in place by a common matrix. b. portion of a trichome projecting from the matrix. c. heterotrophic bacteria attached to *Hydrocoleum* sp. filaments.

III. NITROGEN FIXATION IN ISOLATED CULTURES OF *HYDROCOLEUM*

A. *NifH* phylogeny of *Hydrocoleum*

Using degenerate primers, *nifH* from *Hydrocoleum* cultures was amplified, cloned and sequenced. Multiple clones yielded a single *nifH* sequence, indicating that only one *nifH* gene occurs in the *Hydrocoleum* isolated from mats. Similar *nifH* sequences in the NCBI GenBank are from a wide range of marine environments, including intertidal, near-shore and pelagic locations. Most similar GenBank entries are labeled as “uncultured cyanobacteria” or “uncultured bacteria”. Of those identified to the genus level, *Lyngbya*, *Osillatoria*, and especially*Trichodesmium*, appeared to be most closely related (Fig 23). Microbial mats typically contain proteobacteria, and many bacteria were observed in the mats used for the study. A comparison of the *nifH* sequence from *Hydrocoleum* with reported proteobacterial *nifH* sequences indicated a max identification (NCBI Blast) less than 67 %, while all entries shown in Fig 23 had a max identification higher than 91 %. Thus, the *Hydrocoleum nifH* sequence appears to have little sequence similarity to proteobacterial *nifH*. The “uncultured bacteria” entries in Fig 23 do not indicate if the source of DNA was from cyanobacteria or heterotrophic bacteria. Results described above suggest they may all be from cyanobacteria.

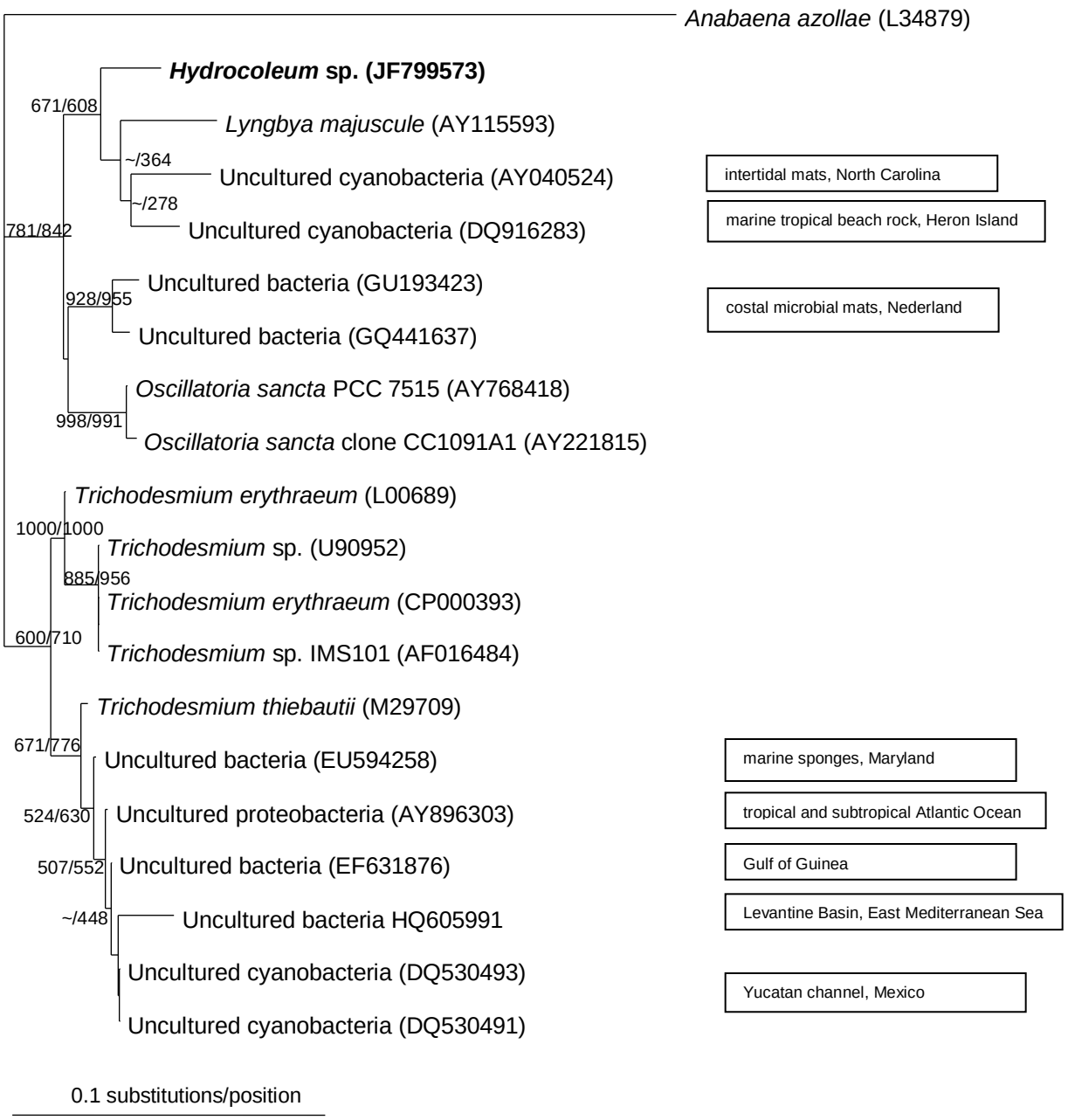


Fig 23. Phylogenetic tree of isolated *Hydrocoleum* sp., based on its *nifH* gene. The unrooted tree used the heterocystous nitrogen-fixing organism *Anabaena azollae* (L34879) as the outgroup. The tree was generated

using both maximum likelihood (ML) and Neighbor joining (NJ) analyses. Numbers at nodes represent bootstrap probabilities of ML and NJ (ML/NJ) in 1000 replicates. Branch lengths are proportional to the number of substitutions per position. “Uncultured cyanobacteria”, “Uncultured bacteria” and “Uncultured proteobacteria” were environmental samples that have not been isolated. Their original locations were labeled in adjacent rectangular boxes.

B. Nitrogen fixation in continuous darkness

Although *Hydrocoleum* could fix nitrogen during a dark period following illumination (see Fig 13), nitrogenase activity was not sustained in continuous darkness (Fig 24). After 12 hr of light, *Hydrocoleum* samples were kept in continuous darkness for 36 hr. Nitrogenase activity went down to near zero by 18 hr into the dark period. Although nitrogenase activity remained low during the rest of the dark period, some activity was observed during hours 24 - 36 (inset of Fig 24). Had the sample been illuminated from hours 12 to 24, this activity would correlate with the second dark period. Thus, the timing of nitrogenase activity may be under diurnal control.

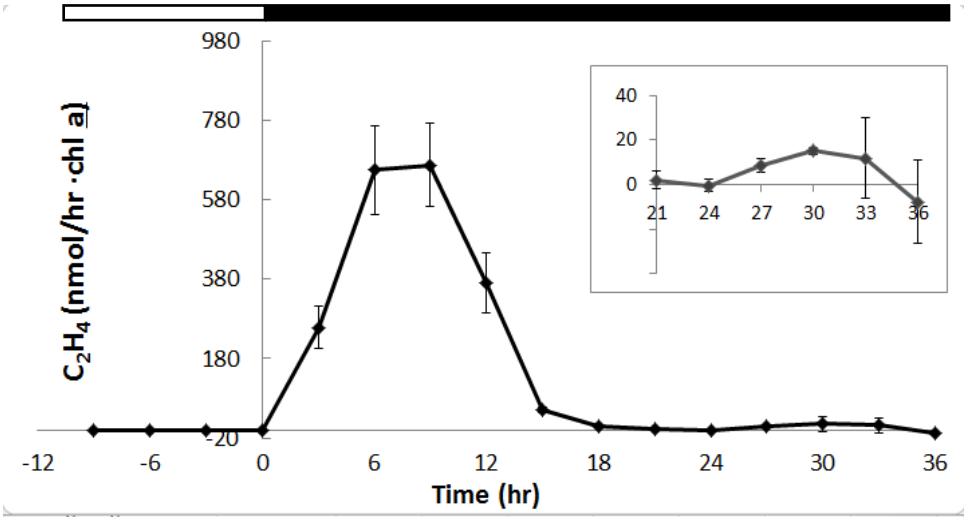


Fig 24. Nitrogenase activity of unialgal *Hydrocoleum* cultures under 36 hr of continuous darkness. Samples used in these measurements were placed in darkness for 12 hr (not shown), then illuminated for 12 hr (shown as -12 hr to 0 hr), and then kept in continuous darkness. The white bar above the plot shows the light period; the black bar shows the dark period. The inset figure shows an expanded view of the activity between the 21rd and 36th hrs. Bars show the range of duplicate measurements (n=2).

The level of *Hydrocoleum nifH* mRNA was measured at intervals between 0 time and 24 hr into a continuous dark period (Fig 25). *NifH* mRNA levels gradually decreased to near zero by 15 hr into the dark period, and remained low through the remainder of the dark period. In contrast, in experiments where samples were illuminated after a 12 hr dark period, mRNA levels increased throughout the 12 hr light period. Thus *nifH* mRNA expression occurs in light,

while most nitrogenase activity occurs during a dark period following illumination.

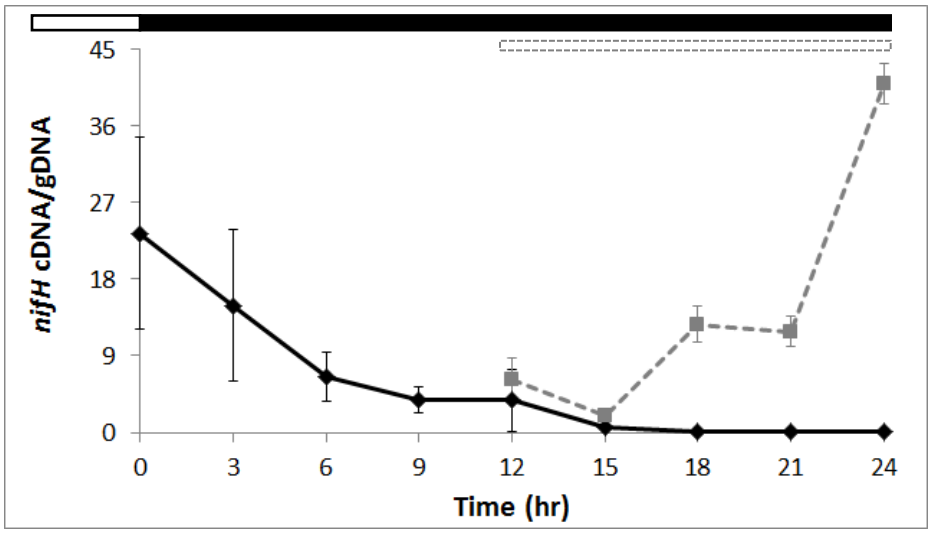


Fig 25. *NifH* mRNA levels during 24-hr dark period. Samples used in these measurements contained unialgal *Hydrocoleum* cultures. They were placed in darkness for 12 hr (not shown), then illuminated for 12 hr, and then kept in continuous darkness (dark line). The grey line shows *nifH* mRNA levels in an experiment where samples were illuminated from hours 12 to 24. The white bars above the plot show light periods; the black bar shows the dark period. Bars show the range of duplicate measurements (n=2).

C. Nitrogen fixation under anaerobic conditions

For most acetylene reduction assays, flasks containing samples were sealed during experiments, but were not flushed with N_2 before the assays, so the O_2 concentration at the beginning of experiments was the same as ambient (~21% O_2). In order to test the effect of O_2 on the nitrogenase activity of *Hydrocoleum* cultures, flasks were flushed with N_2 prior to assays to exclude O_2. No activity was observed during the light period regardless of whether or not O_2 was present or not (Fig 26). However, anaerobic samples that were incubated with 50 µM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) to inhibit photosynthesis (Ramanujam et al. 1981) produced substantial amount of ethylene during the light period (Fig 27). Nitrogenase activity during the subsequent dark period was much lower in the absence of O_2 than when the flasks initially contained ambient O_2 concentration (Fig 26). Therefore, it appears that some O_2 is necessary for dark-dependent nitrogenase activity, perhaps by allowing respiration to generate ATP and reductant. Fig 28 supports this proposal by showing that anaerobic samples with the injection of 4 ml of air into the 20-ml head space at the beginning of the dark period generated higher nitrogenase activity than samples without added O_2.

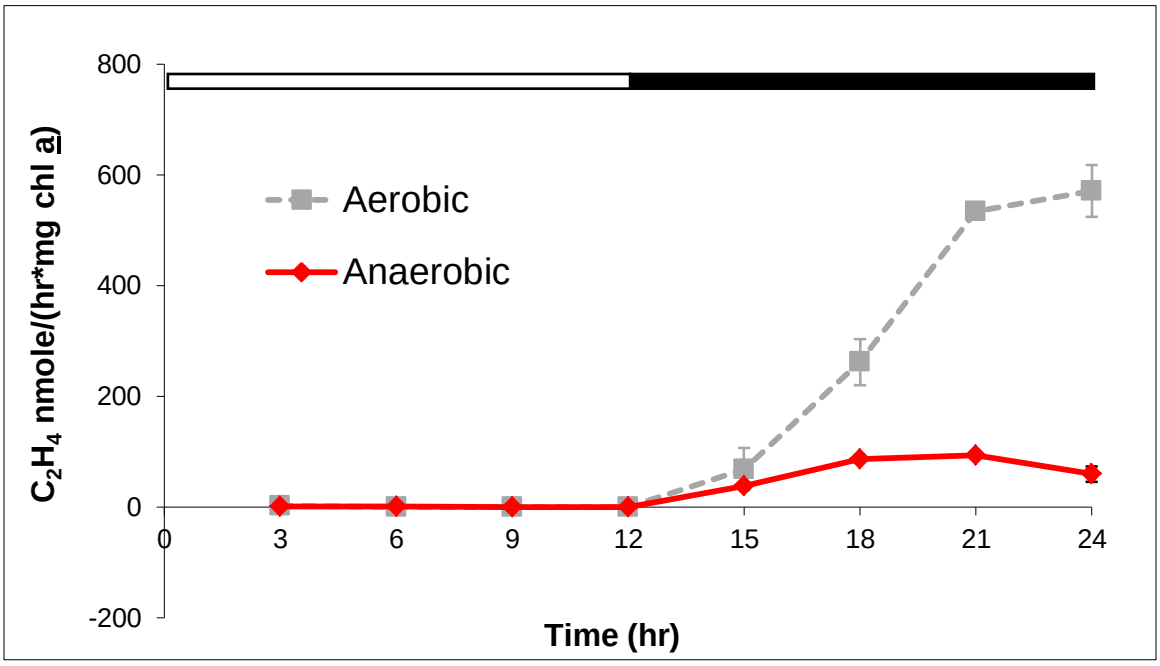


Fig 26. Effect of O_2 concentration on nitrogenase activity in unialgal *Hydrocoleum* cultures. The white bar above the plot shows the light period; the black bar shows the dark period. Bars show the range of duplicate measurements (n=2). No bars are shown where duplicate measurements were virtually identical.

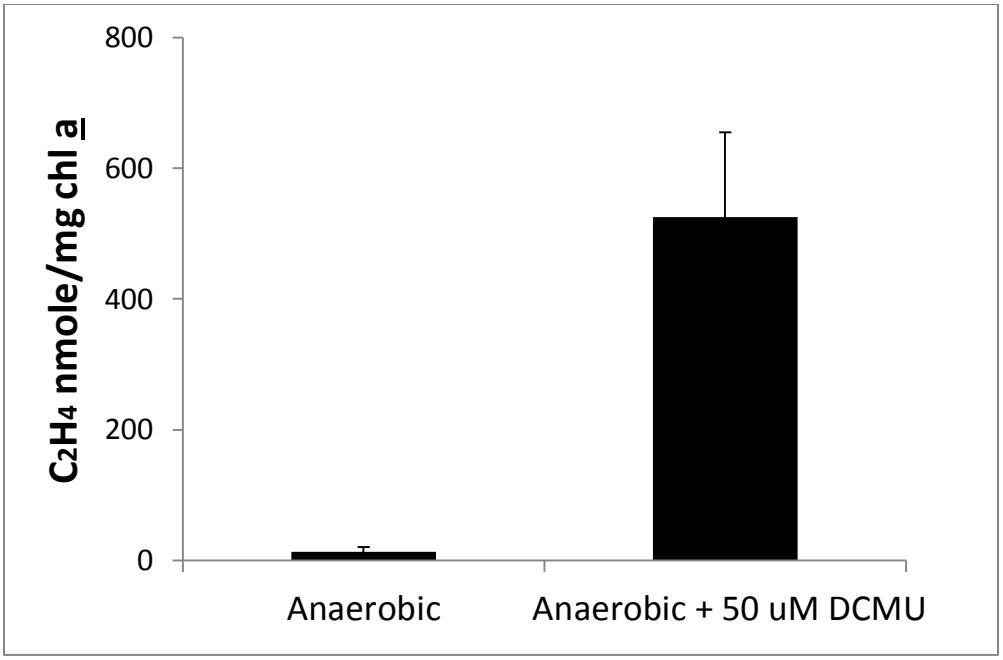


Fig 27. Effect of DCMU on nitrogenase activity of *Hydrocoleum* during the light period. Each bar represents the total amount of ethylene produced during a 12 hr light period. DCMU was added to samples at the beginning of the light period. Bars show the range of duplicate measurements (n=2).

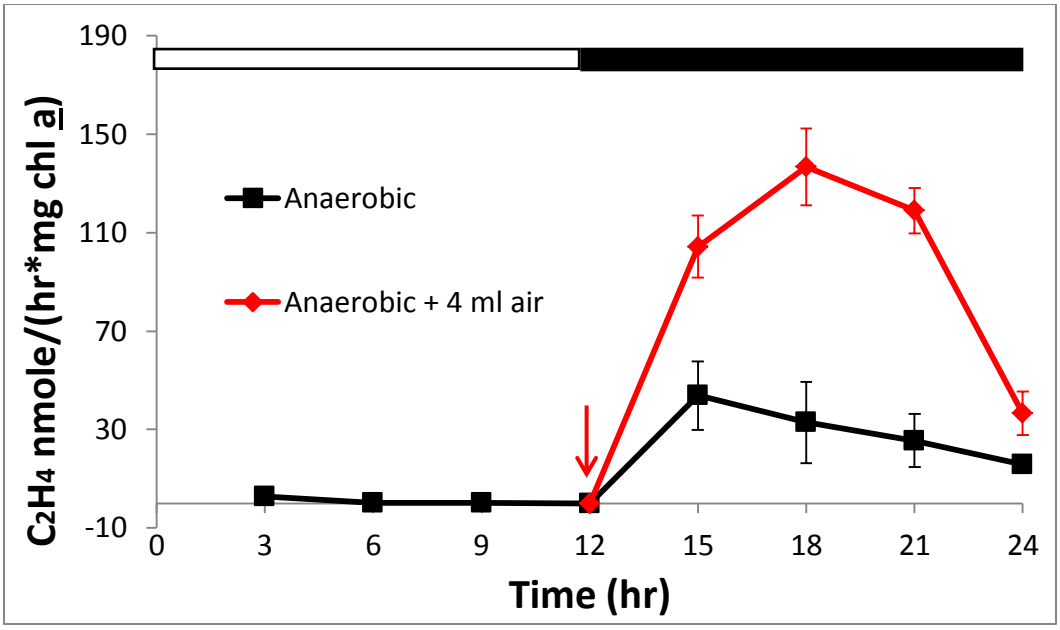


Fig 28. Effect of O_2 on nitrogenase activity of anaerobic *Hydrocoleum* samples during the dark period. The red arrow indicates the time when 4 ml air was injected into anaerobic samples. The white bar above the plot shows the light period; the black bar shows the dark period. Bars show the range of duplicate measurements (n=2).

NifH mRNA levels of *Hydrocoleum* cultures were also influenced by O_2 (Fig 29). Under anaerobic condition, the level of *nifH* mRNA increased in the light then decreased in subsequent darkness. The pattern of *nifH* expression was somewhat similar when samples were kept under ambient atmospheric conditions (~ 21% O_2), although peak activity occurred slightly later, and total activity was much higher, when samples were exposed to ambient air (Fig 29).

Thus both *nifH* mRNA expression and nitrogenase activity (Fig 26) are highest in *Hydrocoleum* cultures that are maintained under aerobic conditions.

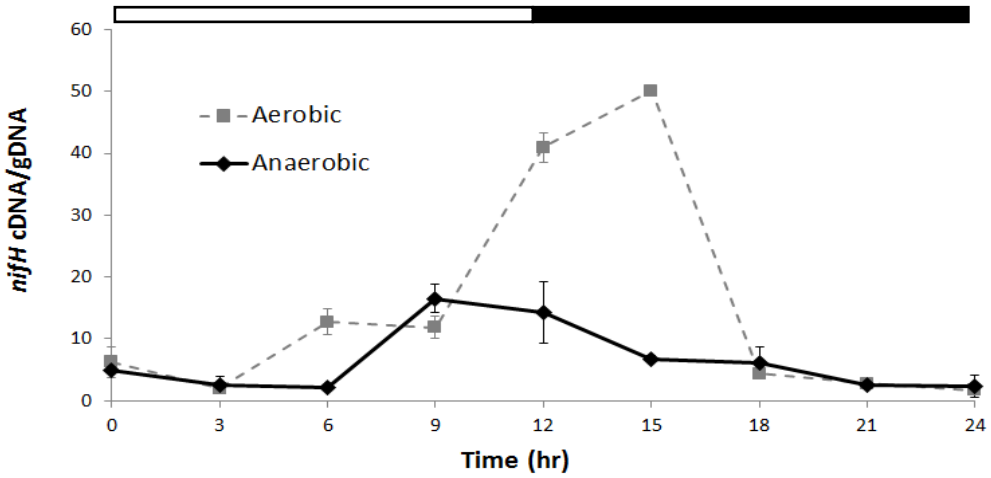


Fig 29. Effect of O_2 on *nifH* mRNA levels of unialgal *Hydrocoleum* cultures. The white bar above the plot shows the light period; the black bar shows the dark period. Bars show the range of duplicate measurements.

D. Nitrogen fixation at various temperatures.

Hydrocoleum cultures were generally maintained at 25 °C in the laboratory and most experiments were conducted at 25 ˚C. For some measurements, cultures were transferred to a different temperature for one week in order to allow them to acclimatize. Nitrogenase activities and *nifH* expression levels were then measured during a diel cycle at the acclimatized temperature. Nitrogenase activities at 20 °C, 30 °C and 35 °C were much lower than were rates at 25 °C (Fig 30). However, the overall pattern of NA was the same at all temperatures.

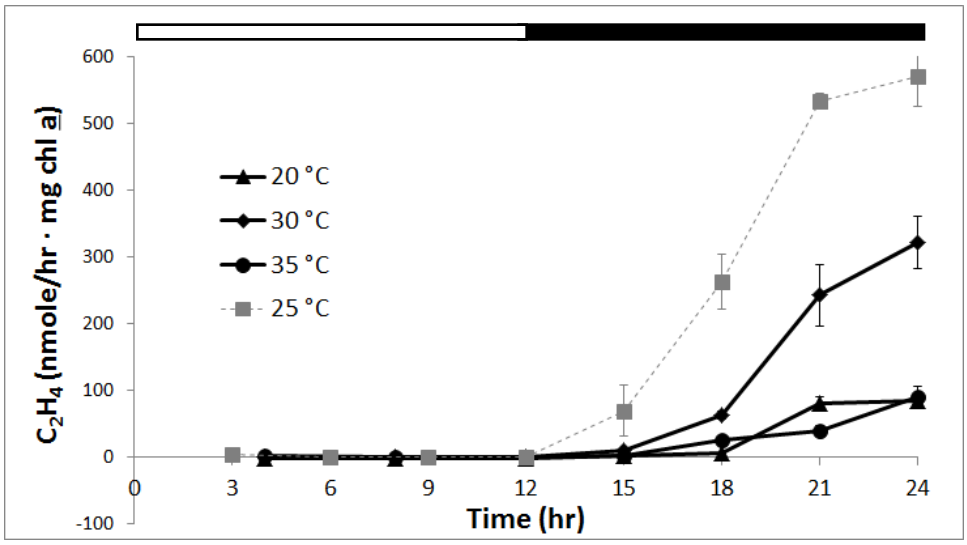


Fig 30. Effect of temperature on the level and diel pattern of nitrogenase activity in unialgal *Hydrocoleum* cultures. The white bar above the plot shows the light period; the black bar shows the dark period. Bars show the range of duplicate measurements.

Both the level and the diel pattern of *nifH* mRNA expression in *Hydrocoleum* cultures were profoundly influenced by temperature. Maximum expression was seen at 25 ˚C. The relative levels of expression as a function of temperature (Fig 31) correlated rather closely to temperature dependence of nitrogenase activity (Fig 31). Peak expression occurred several hours earlier in cultures maintained at 25 ˚C than in cultures maintained at a higher or lower temperature (Fig 31). This contrasts with times during the diel cycle of maximum nitrogenase activity, which were the same at all temperatures (Fig 30).

Regardless of the temperature, *nifH* expression levels approached zero at the end of the dark period of a diel cycle.

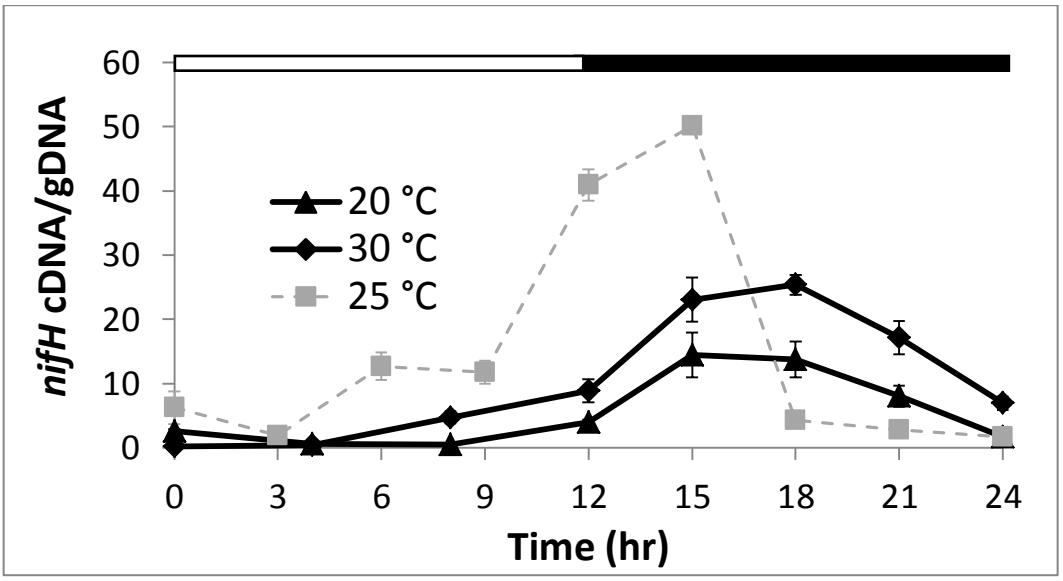


Fig 31. Effect of temperature on the level and diel pattern of *nifH* mRNA expression in unialgal *Hydrocoleum* cultures. The white bar above the plot shows the light period; the black bar shows the dark period. Bars show the range of duplicate measurements.

E. Nitrogen fixation at two different light intensities

Isolated *Hydrocoleum* cultures were maintained under dim light (60-70 $\mu E \cdot m^{-2} \cdot s^{-1}$) in the laboratory. Cultures were acclimatized by placing flasks at a light intensity of ~ 400 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 2 days prior to light-intensity experiments. Acclimatized cultures were then separated into two fractions in separate flasks. One flask was placed under bright light (~ 900 $\mu E \cdot m^{-2} \cdot s^{-1}$); the other was placed under the light intensity (~ 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) normally used for the light period

during acetylene reduction assays. Nitrogenase activities were measured after 2 days of acclimation at the new light intensity. Assays were conducted at the same light intensities as acclimation intensities (Fig 32). Although the diel pattern of nitrogenase activity in *Hydrocoleum* cultures was the same at both light intensities, the dark-mediated nitrogenase activity was significantly (~ 50%) higher in cultures that were subjected to the higher preceding light intensity. Thus, light may provide stored energy and/or reductant for nitrogen fixation during the subsequent dark period.

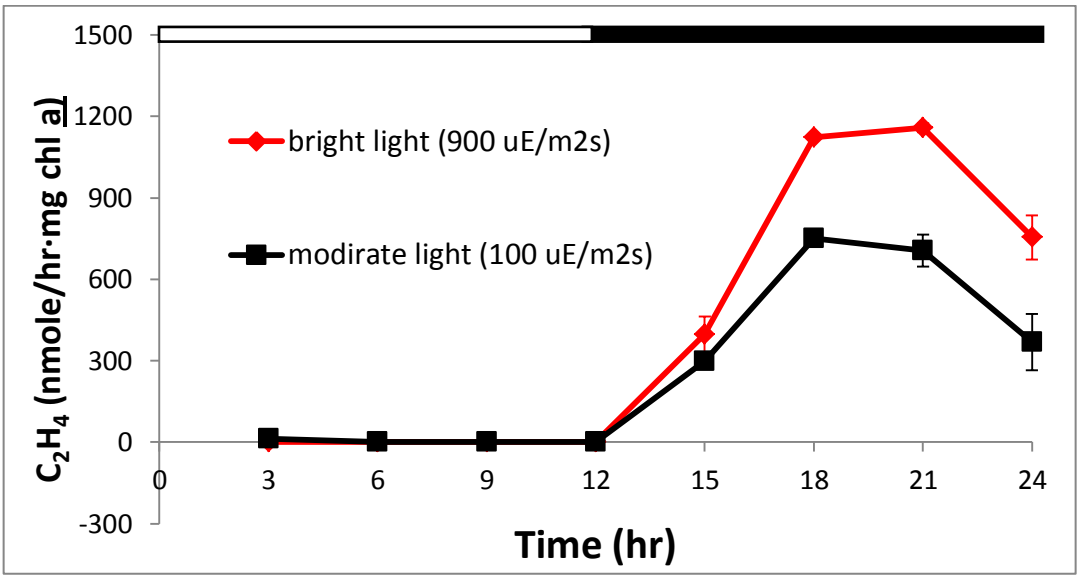


Fig 32. Effect of light intensity on level and diel pattern of nitrogenase activity in unialgal *Hydrocoleum* cultures. The white bar above the plot shows the light period; the black bar shows the dark period. Bars show the range of duplicate measurements.

CHAPTER FOUR: DISCUSSION AND CONCLUSIONS

A. Nitrogen fixation in cyanobacteria-based mats on the South Texas Gulf Coast occurs primarily in *Hydrocoleum*

Hydrocoleum may be the most common mat-associated cyanobacterium of tropical oceans (Abed et al. 2006). Previous studies of *Hydrocoleum*-dominated microbial mats show a pattern of nitrogenase activity that is similar to that of mats used in this study (Charpy 2007). However, no previous comparison of the pattern of nitrogen fixation in intact mats and in isolated *Hydrocoleum* has been reported. One of the two dominant organisms in the South-Texas mats is identified as a *Hydrocoleum*, based on its morphology, its16s rDNA sequence, and its intense coloration that is easily discerned in intact mats. This strain could be isolated in unialgal culture so that its nitrogen-fixation characteristics and growth form could be compared with features of intact mats.

Although various kinds of prokaryotes within microbial mats may contribute to nitrogen fixation of the whole system, results presented here suggest that nitrogen fixation in cyanobacteria-based mats from the South Texas Gulf Coast may occur primarily in a single species of *Hydrocoleum*. Several lines of evidence support that conclusion. (a) There is a direct correlation between the occurrence of *Hydrocoleum* in mats and the ability of these mats to undergo nitrogen fixation (Figs 4 – 7). (b) The diel cycle of nitrogenase activity is similar in mature mats and in isolated cultures of *Hydrocoleum* (Fig 13). (c) The pattern of

expression of the Fe-protein is similar in intact mats and in isolated *Hydrocoleum* (Figs 12, 14).

B. A species-specific *nifH* gene product is a practical tool for monitoring nitrogen fixation of a single species in a community.

Complex communities such as microbial mats form an interacting environment where two or more species may contribute to a single phenotypic characteristic of the community, such as nitrogenase activity. Contributions of a single species to total nitrogen fixation are often difficult to discern, since nitrogenase activity measurements in the entire community only show the sum of activities from all active diazotrophs. Nitrogenase proteins, including MoFe-protein and Fe-protein, are very similar in structure in most diazotrophs, ranging from free-living (e.g. cyanobacteria and proteobacteria) to symbiotic (e.g. *Rhizobia*) organisms. Antibodies raised against nitrogenase of a specific diazotroph are not selective, but can bind to nitrogenase from diverse diazotrophic organisms. Thus, neither enzyme activity nor the proteins themselves are specific enough when an individual organism within a complex community is targeted. Although *nifH* confers the same function in all organisms with active nitrogenase, the large database of *nifH* genes indicates that *nifH* DNA and its corresponding mRNA sequences are distinct for each different species. Therefore, a specific *nifH* sequence may be used as a proxy for nitrogen fixation of the target organism at the transcription level, even when the organism occurs

along with other organisms that also may fix nitrogen in a complex community. A strain of cyanobacteria isolated from a microbial mat may be used to determine the sequence the *nifH* gene which correlates specifically with the strain of interest. Various characteristics of nitrogenase expression and function can also be compared in intact mats and in the isolated organism.

Carefully designed primers that detect *nifH* from only one species are important for selectively monitoring that species in a complex mat community. Primers designed for the target *Hydrocoleum* strain were generated from DNA that was obtained from isolated cultures. Selectivity tests demonstrated that DNA from various diazotrophs was not amplified by the primers unless *Hydrocoleum* DNA was included in the mixture of tested DNA. Of particular note, no DNA isolated from subsurface proto-mats, which contained virtually no *Hydrocoleum*, was amplified by the primers.

The level of *Hydrocoleum nifH* transcripts within whole mats and the upper layer of mats correlate well with the level of *nifH* transcripts in isolated cultures of *Hydrocoleum* (Fig 15). Studies of isolated *Hydrocoleum* cultures indicate that the level of *nifH* transcription can be correlated with nitrogenase activity (Figs 26-29). Therefore, nitrogenase activity (NA) in *Hydrocoleum* within mats may also be similar to that of *Hydrocoleum* in isolated cultures under similar environmental conditions. With that assumption, the data from isolated cultures may be used to interpret the contribution to total nitrogen fixation by *Hydrocoleum* in mats.

The patterns of *Hydrocoleum nifH* expression in mats and in isolated cultures were not precisely coincident, with the peaks of expression occurring at slightly different hours in a diel cycle (Fig 15). Studies of isolated cultures of *Hydrocoleum* indicate that the pattern of *nifH* expression varies somewhat with environmental conditions such as O_2 level (Fig 29) and temperature (Fig 31). These results suggest that although both mats and isolated *Hydrocoleum* cultures were at 25 ˚C and under aerobic conditions at the time of assays, the actual environmental conditions surrounding *Hydrocoleum* in mats may be different from the conditions of *Hydrocoleum* in cultures. For example, metabolic reactions in adjacient organisms may create a localized anaerobic or microaerobic environment surrounding *Hydrocoleum*. The pattern of *nifH* transcription of *Hydrocoleum* in mats is likely to be more similar to the pattern of *nifH* transcription in isolated cultures under anaerobic conditions rather than under aerobic conditions (Figs 15 and 27). The actual microenvironments within mats are complex, with various parameters influenced by multiple species. Therefore, it is hard to know the exact environmental conditions of individual filaments of *Hydrocoleum* within intact mats. However, by comparing the pattern of *nifH* gene transcription in mats with the pattern in isolated cultures under various conditions, it is possible to infer conditions that surround *Hydrocoleum* in mats.

C. Stability of nitrogen fixation with time after collection of mats

Field measurements of nitrogenase activity in mats is preferable since activity may decline or change in character after samples are removed to the laboratory. However, the necessary equipment is not always available. Thus, samples were brought to a greenhouse for maintenance. A previous study showed that the pattern of nitrogenase activity of microbial mats did not change significantly after collection, providing that the mats were collected and analyzed at 20 ˚C or 30 ˚C. Most mats used in this study were collected at 25 ˚C, and were maintained in a greenhouse at approximately the same temperature. In most cases, acetylene reduction activities were measured within 2 days after sample collection, but the pattern and level of nitrogenase activity was relatively stable for much longer period of time (at least 3 weeks) (Fig. 10). Thus, laboratory measurements relating to nitrogenase activities in mats that were maintained in the greenhouse are likely very similar to those which occur in nature.

D. Source of nitrogenase activity in isolated *Hydrocoleum* cultures

Amplification of *nifH* genes identified in microbial mats from many locations show that various cyanobacteria and proteobacteria are capable of fixing nitrogen (Zehr et al. 2003). Nitrogenase activity of unialgal, but non-axenic, strains of cyanobacteria that are isolated from environmental samples might come from the cyanobacterium or from hetertrophic bacteria in the culture. Scanning electron microscopic images of isolated *Hydrocoleum* (Fig 22) show

that bacteria are tightly attached to their sheaths. Two methods were used to address the question of which organism in *Hydrocoleum* isolated cultures demonstrated nitrogenase activity. (a). Amplification of *nifH* from total DNA extracted from *Hydrocoleum* isolated cultures using degenerate primers yielded a single *nifH* sequence. This *nifH* sequence shows ≥91% identity to sequences from other cyanobacteria, such as *Lyngbya*, *Oscillatoria* and *Trichodesmium*, but ≤ 67% identity to *nifH* sequences from typical nitrogen fixing proteobacteria. Since only one *nifH* gene was identified in unialgal *Hydrocoleum* cultures, it seems highly likely that nitrogenase activity in these cultures is expressed exclusively from the cyanobacterium. (b). The pattern of nitrogenase activity in isolated *Hydrocoleum* cultures during diurnal illumination matches the typical NA pattern of most non-heterocystous cyanobacteria. Since the nitrogenase enzyme complex is very sensitive to O_2, most non-heterocystous cyanobacteria temporally separate nitrogen fixation from oxygenic photosynthesis, so that NA is only detected in darkness. In contrast, nitrogenase activity of proteobacteria is not directly affected by illumination. It is possible that nitrogenase of heterotrophic bacteria in proximity to phototrophs would be suppressed in light due to oxygenic photosynthesis, although some free-living bacteria don't require anaerobic conditions to fix nitrogen. Also, previous studies have shown that rates of nitrogenase activities of some proteobacteria are relatively constant over time (Bazylinski et al. 2000), while these results (Fig 13) show that NA of *Hydrocoleum* cultures and mats continually changed during the dark period.

These observations together strongly indicate that nitrogenase activities measured in isolated *Hydrocoleum* cultures were from the cyanobacterium, not from associated heterotrophic bacteria.

It is possible that although *Hydrocoleum* contains the active *nif* genes, it and the bacteria attaching to it form a consortium that is required for nitrogen fixation, as has been described previously (Bolhuis et al., 2010; Li et al., 2010). The previous work showed that although the cyanobacterium contained the *nifH* gene, axenic cultures could not fix nitrogen. Nitrogenase activity was detected only when the cyanobacterium retained adhering heterotrophic bacteria (Li et al., 2010). Despite many attempts, using various methods, no axenic cultures of *Hydrocoleum* could be obtained. Perhaps the adhering bacteria are essential to some function of *Hydrocoleum*.

E. Factors that affect nitrogen fixation in *Hydrocoleum* cultures

As a non-heterocystous cyanobacterium, *Hydrocoleum* cells separate oxygenic photosynthesis and nitrogen fixation in time. Nitrogenase activity occurs only in darkness (Fig 13). But at the levels of transcription and translation, *nifH* gene expressions of *Hydrocoleum* were not strictly nocturnal. The number of *nifH* transcripts gradually increased throughout the entire light period, into the first few hours of the following dark period, and then decreased to near zero during the remainder the dark period (Fig 15). The concentration of Fe-protein was low at the beginning of light period, but it increased substantially toward the end of a

light period, and it remained high throughout the following dark period (Fig 14). These results suggest that transcription and translation of *nifH*, and probably other nitrogenase structural genes (*nifD* and *nifK*), are not totally separated temporally from photosynthesis. Although the activity of nitrogenase is extremely sensitive to O_2, mRNA and protein synthesis can occur at the same time as photosynthesis (Figs 14, 15). In fact, their levels of expression may be under diurnal control. A light:dark cycle appears to be required for *nifH* gene expression (Fig 25) . The level of *nifH* transcripts does not increase without a light period in a diel cycle. It is possible that although nitrogenase proteins are irreversibly inhibited at the beginning of light period (Berman-Frank et al. 2001), new proteins are also synthesized during the same light period. That would explain the variation of protein expression with time during illumination (Fig 14). *Hydrocoleum* under continuous darkness may stop synthesizing new nitrogenase mRNAs and proteins. Previously synthesized nitrogenase enzymes remain stable in cells, since there is no photosynthetically generated O_2 to destroy them. Factors such as ADP·AlF_4^- and sugars can help stabilizing nitrogenase in cells during the dark period (Ernst et al. 1984, Schindelin et al. 1997). However, stored energy and reductant may be mostly consumed within 18 hrs, and respiration may cease due to exhaustion of sugars, thus, nitrogen fixation is halted because of both energy limitation and loss of stability due to sugar depletion (Fig 24). No significant activity of nitrogenase is expected to occur if *Hydrocoleum* is maintained in continuous darkness. A small peak of nitrogenase activity was

observed between hours 24 and 36 in darkness. This observation has at least two possible explanations: (a). Nitrogen fixation of *Hydrocoleum* is weakly regulated by a circadian rhythm, as is observed in other non-heterocystous cyanobacteria (Huang et al. 1990, Chen et al. 1996). (b). Another metabolic pathway that generates energy and reductant, such as respiration, is controlled by a circadian rhythm (Kondo et al. 1993, Cerveny and Nedbal 2009). If active during hours 24 – 36, then energy and reductant would be available for nitrogen fixation. Either way, cells may not have much stored energy or reductant at 24 hr of continuous darkness since no photosynthesis occurs. The low nitrogenase activity during 24 – 36 hr in comparison to activities during a diel cycle (Fig 24), might be explained by a limiting supply of energy and/or reductant.

Nitrogenase is sensitive to O_2, so nitrogen fixation in many diazotrophs, including non-heterocystous cyanobacteria occurs best under anaerobic conditions (Fay 1992, Li et al. 2010). Elimination of ambient O_2 does not form an absolute anaerobic environment in cyanobacteria cultures since photosynthesis generates a significant amount of O_2. This probably explains total inhibition of nitrogenase in *Hydrocoleum* cultures during illumination, even in assays that are started under anaerobic conditions (Fig 26). Adding DCMU into the samples inhibited photosystem II-mediated O_2 evolution. The environment then approached anaerobic and an enhanced amount of ethylene was produced (Fig 27).

Unexpectedly, nitrogenase activity during a dark period following illumination was much higher when assays were conducted in the presence of O_2 than when the culture was placed under anaerobic conditions during a diel cycle (Fig 26). Injection of some O_2 into anaerobic samples at the beginning of dark period increased nitrogenase activities during the entire dark period (Fig 28). Those observations might be explained by assuming that light-generation of energy that would be available for nitrogenase activity is limited in quantity and quickly becomes depleted during the subsequent dark period. Dark respiration gradually depletes O_2 in cells that are under an anaerobic or microaerobic environment, thus stabilizing functional nitrogenase. Respiration also supplies energy, some of which can be used directly for nitrogen fixation. Respiration is dependent on O_2, which is limiting under microaerobic conditions. Thus cultures that are prepared in an anaerobic environment prior to a diel cycle may remain in an O_2-limited environment, even after 12 hr of photosynthesis, thereby limiting the extent of respiration during the subsequent dark period. This limited respiration would be expected to limit the amount of ATP available for nitrogen fixation.

Previous studies using non-heterocystous cyanobacteria *Gloeothece* and *Oscillatoria*, showed that NA reached the highest levels immediately after illumination, and then declined to zero by the end of the dark period (Stal and Krumbein 1985). It was concluded that the peak activity at the beginning of dark period was due to photosynthetically derived energy that was available for nitrogen fixation. However, in *Hydrocoleum*, the nitrogenase activity increased

gradually in the dark period, and reached the highest level at, or near, the end of dark period (Figs 13 and 32). For *Hydrocoleum* cultures maintained aerobically, energy is expected to be sufficient for nitrogenase activity at the beginning of a dark period, but the O_2 concentration in cells is also high. Thus, nitrogenase proteins may still be partially inhibited. Nitrogenase activity would then increase as O_2 is consumed and reductant becomes available via respiration. Activity is expected to continue in darkness as long as sufficient energy and reductant are available, providing that diurnal regulatory effects do not modulate activity. The time of maximum activity in the dark period was somewhat variable, even in replicate experiments, although the general trend was always the same. This variability is probably explained by differences in the metabolic state of different cultures at the beginning of the experiments. For example, older cultures may have more stored energy reserves, thereby allowing more sustained period of dark respiration.

F. *Hydrocoleum* as a secondary founder in the formation and development of mats

The theory of "joint ventures" (van Gemerden 1993), states that microbial mats have a tendency to spread both horizontally and upwards whenever moisture is available. Various morphologies of mats observed during collection trips for this study support the joint venture theory. These observations also suggest a hypothesis to explain the successional development and seasonal

differences in mats located along the South Texas Gulf Coast. During dry seasons, a green stratum composed of mostly *Microcoleus*-like cyanobacteria is submerged several mm beneath the surface of the sand, where it retains moisture and receives minimal sunlight. This nearly unialgal and very fragile layer is called a subsurface proto-mat because it does not have the physical integrity, laminate design or community complexity of mats that appear on the surface, but may be the basis for forming surface-exposed mats (Fig 6). The community of *Microcoleus* and associated heterotrophic bacteria grow upward and soon reaches the surface during wet seasons. Motility of *Microcoleus* may hasten this process since a previous study demonstrated that sub-surface *Microcoleus* in wet inshore marine mudflats is able to migrate to the surface by gliding motion (Whale and Walsby 1984). The green layer eventually appears on the surface. The moisture level often varies considerably over the surfaces of beaches, resulting in areas that differ in surface moisture content, especially during times of intermittent rains (Fig 3a). Broad areas of beaches remain moist during periods of frequent rainfalls, resulting in the appearance of large areas of continuous surface mats. As the mat layer expands upward during sustained periods of sufficient moisture, older components of the mat organic matter become buried deeper and deeper where they receive less sunlight and oxygen, and eventually became degraded. The partially degraded organic matter retains EPS that was secreted previously, holding sand particles and organic debris together into a bottom layer of the mat. This entire coherent structure observed in

rainy seasons, consisting of a green upper layer and a thicker black lower layer, is called a mature mat. It is morphologically the most complex organic structure that was seen at the collection site (Fig 4). This hypothesized successional development of mats is proposed to be reversible. When areas that contain surface mats lose moisture during dry periods, surface organisms gradually die and deteriorate while motile cyanobacteria such as *Microcoleus* migrate to subsurface locations where moisture is retained. The diversity of organisms in mats is expected to decrease during these harsh subsurface environmental conditions. As organic debris is consumed, the subsurface *Microcoleus* form a coherent green subsurface stratum (a proto-mat).

The observation that cultures of isolated *Hydrocoleum* form mat-like structures in undisturbed flasks suggests that they might be early colonizers of intertidal sand, providing a framework for colonization by other organisms into an incipient mat. However, proto-mats contain no detectable *Hydrocoleum*. Thus, it seems more likely that *Microcoleus* is the primary colonizer, forming a fragile matrix for recruitment of other organisms. The inclusion of *Hydrocoleum* as a secondary founder when this community approaches the surface provides additional structure and coherence, attracts additional organisims, and facilitates the fixation and assimilation of N_2. *Lyngbya*, and *Oscillatoria* are proposed to act as soil binders and stabilizers in mats from other locations (Gerdes 2010), and may perform functions similar to those performed by *Hydrocoleum* in mats of the South Texas coast.

Appendix I

Sequences of genes identified from *Hydrocoleum*

16s rDNA (partial) (GeneBank access number JF799752)

GTGGCGGACGGGTGAGTAACGCGTGAGAATCTGCCTTCAGGTCGGGGACA
ACAGAGGGAAACTTCTGCTAATCCCGGATGAGCCTTTGGGTAAAAGATTAAT
TGCCTGGAGATGAGCTCGCGTCCGATTAGCTAGTTGGTAGGGTAAAAGCTT
ACCAAGGCAACGATCGGTAGCTGGTCTGAGAGGATGAGCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTC
CGCAATGGGCGAAAGCCTGACGGAGCAAGACCGCGTGGGGGAGGAAGGC
TCTAGGGTTGTAAACCCCTTTTCTTTGGGAAGAAGTTCTGACGGTACCAAAG
GAATCAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGAG
GCAAGCGTTATCCGGAATGATTGGGCGTAAAGCGTCCGCAGGTGGCAATGT
AAGTCTGCTGTCAAAACCCAGGGCTCAACTCTGGTTAGGCAGTGGAAACTA
CATAGCTAGAGTTCGGTAGGGGCAAAGGGAATTCCCGGTGTAGCGGTGAA
ATGCGTAGAGATCGGGAAGAACATCGGTGGCGAAAGCGCTTTGCTAGACC
GAAACTGACACTCAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATAC
CCCAGTAGTCCTAGCTGTAAACGATGGATACTAGGTGTTGCCTGTATCGAC
CCGGGCAGTGCCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGC
ACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGGCTTGACAT
GTCGCGAACCTAGGGAAAACTCTGGGGTGCCTTCGGGAGCGCGAACACAG
GTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTCGTTTTAGTTCCATCATTTAGTTGGGAACTCTAAA
AGACTG

nifH gene (partial) (GeneBank access number JF799753)

TGTGATCCGAAAGCTGACTCTACTCGTCTAATCTTAAACGCTAAAGCTCAAA
CTACTGTACTTCACGTTGCTGCAGAACTCGGTGCAGTTGAAGACGTAGAAC
TAGAGGAAGTATTGAAGACTGGTTTCGCTGGTATCAAGTGCGTTGAGTCTG
GTGGTCCTGAGCCTGGAGTAGGTTGTGCAGGTCGTGGTATTATCACAGCTA
TCAACTTCCTAGAAGAAGAAGGTGCTTATACCGATCTAGATTTCGTAAGTTA
TGACGTACTAGGTGACGTTGTTTGCGGTG

Appendix II

Sequences of genes identified from MF2 and MF3

MF2 16s rDNA

TGCAAGTCGAACGGACTCTTCGGAGTCAGTGGCGGACGGGTGAGTAACGC
GTGAGAATCTGCCTTCAGGACGGGGACAACAGTTGGAAACGACTGCTAATA
CCCGATGTGCCGAGAGGTGAAAAGGTGAATTCCGCCTGAAGAGGAGCTCG
CGTCCGATTAGCTAGTTGGTGAGGTAAAAGCTTACCAAGGCGACGATCGGT
AGCTGGTCTGAGAGGATGAGCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCT
GACGGAGCAACGCCGCGTGGGGGAAGAAGGTTTTTGGATTGTAAACCCCTT
TTCTCAGGGAAGAAGATCTGACGGTACCTGAGGAATCAGCCTCGGCTAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGAGGCAAGCGTTATCCGGAATC
ATTGGGCGTAAAGCGTTCGTAGGCGGCTGTTCAAGTCTGCTGTCAAAGACC
GAGGCTCAACCTCGGGTCGGCAGTGGAAACTGGATAGCTAGAGGTCGGTA
GGGGCAAAGGGAATTCCCAGTGTAGCGGTGAAATGCGTAGATATTGGGAA
GAACACCGGTGGCGAAAGCGCTTTGCTGGACCGAACCTGACGCTGAGGGA
CGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTA
AACGATGGAGACTAGGTGTTGCCTGTATCGACCCGGGCAGTGCCGAAGCC
AACGCGTTAAGTCTCCCGCCTGGGGGAGTACGCACGCAAGTGTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGANTATGTNGGTTTAATTCG
ATGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCGCGAATCTCGGCGA
AAGTCGGGAGTGCCTACGGGAACGCGGAGACAGGTGGNGCATGGCTGTC
GTCAGCTCGTGTCG

MF3 16s rDNA

AACGCAGTCTTTCGGGACTGAGTGGCGAACGGGTGAGTAACGCGTGAGGA
TCTGCCCTTAGGTGGGGGACAACCGTTGGAAACGACGGCTAATACCGCATA
TGGCGAGAGCTAAAAGCTTAATGTGCCTGAGGATGAACTCGCGTCTGATTA
GCTAGTTGGTGAAGTAACGGTTTACCAAGGCGACGATCAGTAGCTGGTCTG
AGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATTTTCCGCAATGGGGGCAACCCTGACGGAGCAA
CGCCGCNNGGGGGAGGAATGTCTGTGGATTGTAAACCCCTTTTCTCAGGGA
AGAAGTTCTGACGGTACCTGAGGAATCAGCATCGGCTAACTCCGTGCCAGC
AGCCGCGGTAAGACGGAGGATGCAAGCGTTATCCGGAATTATTGGGCGTAA
AGCGTCCGTAGGCGGTTTATCAAGTCTGTCGTTAAAGACTGCAGCTTAACTG
TGGGCGAGCGGTGGAAACTGATTAACTAGAGTGTGGTAGGGGTAGAGGGA
ATTCCCAGTGTAGCGGTGAAATGCGTAGATATTGGGAAGAACACCAGTGGC
GAAGGCGCTNTACTGGGCCACAACTGACGCTGAGGGACGAAAGCTAGGGG
AGCGAAAGGGATTAGATACCCCTGTAGTCCTAGCTGTAAACGATGGATACT
AGGTGTTGCGCGTATCGACCCGTGCAGTACCGCAGCCAACGCGATAAGTAT
CCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCAGGGCTTGACATCCCTCGAATCCTCTTGAAAGAGAGGAGTGCCT
TCGGGAGCGAGGAGACAGGTGGTGCATGGCTGTCGTCANNTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCACGTCCTTAGTTGCCA
TCATTCAGTTGGGCACTCTGGGGAGACTGCCGGGGACAACTCGGAGGAAG
GTGTGGATGACGTCAAGTCATCATGCCCCTTACGTTCTGGGCTACACACGT
ACTACAATGCTTCGGACAAAGGGCAGCGAGCGCGCGAGT

Appendix III

Primers used in the experiments

16s rDNA

Forward: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse: 5'-AAGGAGGTGATCCAGCCGGA-3'

nifH degenerate

Forward: 5'-TGYGAYCCNAARGCNGA-3'

Reverse: 5'-ADNGCCATCATYTCNCC-3'

Y=C, T

N=A, C, T, G

R=A, G

D=A, G, T

nifH primers specific for *Hydrocoleum*:

Set 1: Forward: 5'-GAGGAAGTATTGAAGACTGGTTTC-3'

Reverse: 5'-CGACCTGCACAACCTAC-3'

Set 2: Forward: 5'-GCTGGTATCAAGTGCGTTGAGT-3'

Reverse: 5'-CCACGACCTGCACAACCTACT-3'

TaqMan MGB probe: FAM 5'-TGGTGGTCCTGAGCC-3'

Appendix IV

Primer specificity test for *Hydrocoleum nifH* gene

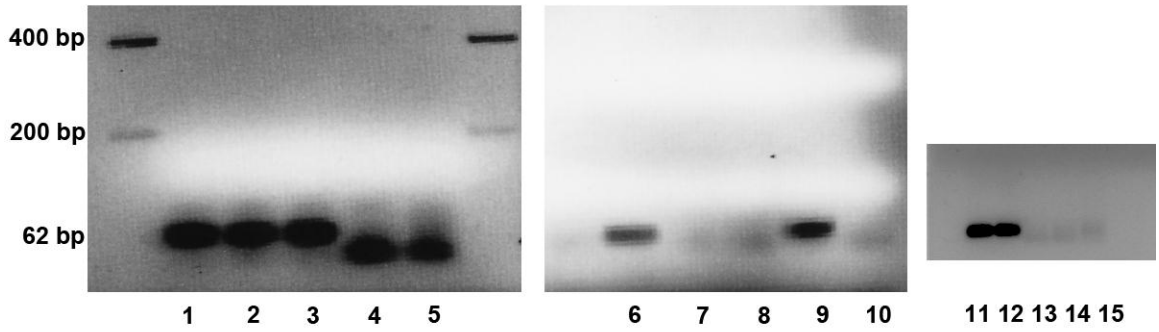


Fig A1. Demonstration of specificity of primers (see Materials and Methods) designed to amplify a 62-base-pair *nifH* partial sequence of *Hydrocoleum*. Lane numbers indicate organisms from which DNA was isolated for *nifH* amplification and observation on an argaose gel. Approximately the same amount of DNA from each organism was placed into the thermal cycler for each amplification.

Lane 1, 9, 11:	*Hydrocoleum*
Lane 2, 12:	Mature mats
Lane 3:	*Hydrocoleum*, MF2, MF3
Lane 6:	*Hydrocoleum*, *Nostoc* (UTEX 1037, UTEX 2210), *Anabaena* (UTEX 1448, UTEX 2576)
Lane 4*:	MF2, MF3
Lane 7:	*Nostoc* (UTEX 1037, UTEX 2210)
Lane 8:	*Anabaena* (UTEX 1448 ,UTEX 2576)
Lane 14, 15:	Proto-mats
Lane 5*, 10, 13:	Negative control (ddH_2O)

*: No actual sequence detected. Bands may be primer dimers and dNTPs.

Appendix V

Calculation of nitrogenase activity in acetylene reduction assays

Acetylene gas was produced based on the reaction:

$$CaC_2 + 2H_2O = C_2H_2 + Ca(OH)_2$$

The C_2H_2 gas was trapped in a gas bulb and then 4.0 ml of gas was taken out by a gas-tight syringe and injected into sample vessels. Reagent grade ethylene gas was purchased from Matheson Tri-Gas with a concentration of 1,000 ppm (parts per million). Data that are directly read from the GC are the values of peak areas for C_2H_4 and C_2H_2 respectively. In order to convert GC reads into C_2H_4 production of samples, a standard measurement was performed by first placing 4.0 ml of C_2H_2 and 1.0 ml of C_2H_4 into a sealed vessel containing no sample, and then, removing 100 µl of mixed gas from the vessel using a gas-tight syringe to inject into the GC.

Calculations:

1.0 ml C_2H_4 injection contains 1000/1000000 = 0.001 ml C_2H_4 (1000 ppm)
Amount of C_2H_4 in the vessel: 0.001/22.4/1000 = 0.0446 mole = 44.6 nmole *

The volume of gas in the vessel is V_0 ml, so the amount of C_2H_4 in a 100 µl injection is:

$$44.6 \text{ X } 0.1 / V_0 = 4.46 / V_0 \text{ nmole}$$

The value of the C_2H_4 peak area for the standard is A_s, and the value of C_2H_2 peak area for the standard is B_s. The ratio of C_2H_4/C_2H_2 (R_s) = A_s/ B_s. The amount of C_2H_2 in an injection is C. Then:

$$R_s = A_s / B_s = (4.46 / V_0) / C$$

$$C = (4.46 / V_0) / R_s$$

C is considered as a constant since during a diel cycle, no more than 2% of the initial C_2H_2 was converted to C_2H_4 in any experiment.

When $A_s = 59.65884$, $B_s = 201287$, then:

$$C = (4.46 / V_0) / (88.33072 / 298065) = 15081.08 / V_0$$

For each GC injection, peak values are A_t for C_2H_4 and B_t for C_2H_2. The total amount of C_2H_4 in the vessel is E_t. Then:

$$R_t = A_t / B_t \text{ and:}$$

$$E_t = R_t \text{ X } C \text{ X } V_0 = (A_t / B_t) \text{ X } (15081.08 / V_0) \text{ X } V_0 = 15081.08 \text{ X } A_t / B_t$$

Injection times for two successive samples are designated as t_1 and t_2, generally spaced 3 hr apart.

The C_2H_4 production from t_1 to t_2 is:

$$E_{t2}\text{-}E_{t1} = 15081.08 \text{ X } (A_{t2} / B_{t2} – A_{t1} / B_{t2})$$

The average rate of ethylene production (as nitrogenase activity, = NA) from time t_1 to t_2 (plotted at time t_2) is:

$$NA_{t2} = (E_{t2}\text{-}E_{t1}) / (t_2 – t_1) = 15081.08 \text{ X } (A_{t2} / B_{t2} – A_{t1} / B_{t2}) / (t_2 – t_1)$$

For example:

At time zero: $A_0 = 5.72716$, $B_0 = 295384$

At time 3-hour: $A_3 = 215.96234$, $B_3 = 298409$

NA at 3-hour: NA = 15081.08 X (5.72716/295384 – 215.96234/298409) / 3
= 15081.08 X 0.000234775
= 3.5403 nmole C_2H_4 / hour

NA data were normalized to mg Chl a in final calculations.

* gas is treaded as ideal (22.4 L/mole).

Appendix VI

Real-time PCR

1. Standard plasmid

A partial sequence of *Hydrocoleum nifH* (286 bp) was inserted into a TOPO TA cloning® vector (pCR®4-TOPO®, 3956 bp) and transformed into *E. coli* cells. The total length of the plasmid extracted from *E. coli* cells was 4242 bp (286 + 3956). Serial dilutions of this plasmid were used as standards in real-time PCR.

Average dsDNA MW = 660 X N g/mole (N is the number of base pairs).

The approximate MW of this plasmid is: 660 X 4242 = 2.79972 X 10^6 g/mole.

The original plasmid solution was diluted into a working solution of 3 X10^6 plasmid copies / 2 µl. Then the working solution was serially diluted 4 times (each dilution 10X).

2. Dynamic range testing for RNA concentration

Serial dilutions of RNA were tested by real-time PCR. A range of optimal concentrations was found. Within this range, there was a liner relationship between Log (RNA concentration) and threshold cycle number (C_t) when the efficiency of real time PCR was near 100%. RNA samples were diluted before reverse transcription because inhibitions at too high a concentration significantly decreased the efficiency of real-time RNA.

An example of optimal concentration tests:

5 of 5-fold dilutions of a RNA sample were prepared:

Dilutions	B (5X)	C (25X)	D (125X)	E (625X)	F (3125X)	G (15625X)
Relative concentration	3125a	625a	125a	25a	5a	a

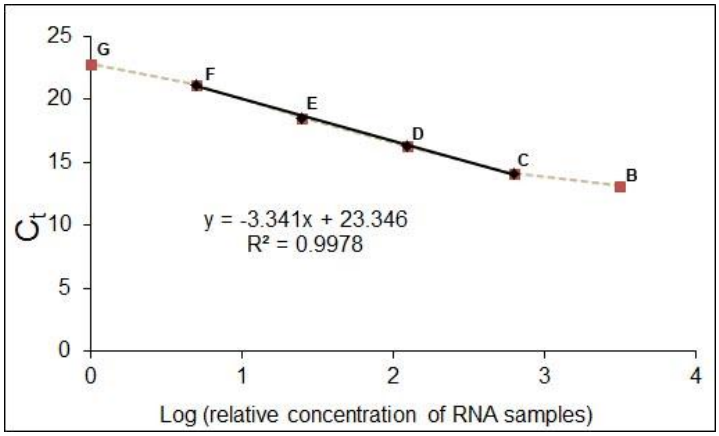


Fig A2. Dynamic range testing for a RNA sample

A linear relationship was formed between dilution C and dilution F for this RNA sample (Fig A2). The optimal concentrations of this RNA in real time PCR were from 1/5 to 1/625 of the original concentration. Therefore, other RNA preparations extracted from the same kinds of sample (the same organism, prepared in the same way prior to PCR) and at similar original concentrations were diluted 125 times before reverse transcription and then amplified by real-time PCR.

3. Dynamic range testing for DNA concentration

The testing for DNA was similar to the testing for RNA described above. Serial dilutions of a DNA sample were prepared and amplified by real-time PCR. A linear relationship between Log (DNA concentration) and C_t occurred at a range of concentrations. The 260/230 ratio of DNA affected the efficiency of real time PCR. When the ratio reached 1.8 or above, the efficiency approched 100% for dilutions in the optimal range of concentrations.

4. Calculations of the cDNA/gDNA ratio

A cDNA and a genomic DNA sample that were used in a ratio calculation (see Materials and Methods) were amplified in the same run. Samples from the same

diel cycle were also amplified in that same run. Three reactions were performed for each sample of cDNA or gDNA, and the average C_t of triplicates was calculated for each sample after amplification. Standards with 5 different concentrations as well as negative controls (reactions without cDNA and DNA) were prepared for each plate.

Calculations:

An equation based on data from standards:

$C_t = - AX + B$

(A and B are determined by data of standards)

$Y = (B – C_t) / A$

$X = \text{Log}Y$ (Y is the initial copy number of the sample)

For a cDNA sample: the average C_t is C_{tr}, and the initial copy number is Y_r.

For a gDNA sample: the average C_t is C_{td}, and the initial copy number is Y_d.

$\text{Log}Y_r = (B - C_{tr}) / A$

$\text{Log}Y_d = (B - C_{td}) / A$

$\text{Log}Y_r - \text{Log}Y_d = (C_{td} - C_{tr}) / A$

$\text{Log} (Y_r / Y_d) = (C_{td} - C_{tr}) / A$

(Y_r / Y_d is the ratio of cDNA / gDNA in reactions)

$(Y_r / Y_d) = 10^{[(Ctd - Ctr) / A]}$

For example: if A = 3.30, C_{td} = 26.70682, C_{tr} = 19.15629

In reactions: $(Y_r / Y_d) = 10^{(26.70682-19.15629)/3.30} = 195.82$

In original RNA and DNA samples, the ratio was modified according to dilution factors in each step prior to real-time PCR. If the RNA was diluted 125 times, and the DNA was diluted 1000 times, the ratio for the original samples is:

195.82 X 125 / 1000 = 24.48

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